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Efficacy of Seed Treatments, Microbial and Biochemical Pesticides for Managing Early Tan Spot and Stripe Rust Diseases of Wheat

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EFFICACY OF SEED TREATMENTS, MICROBIAL AND BIOCHEMICAL PESTICIDES FOR MANAGING EARLY TAN SPOT AND STRIPE RUST DISEASES OF WHEAT

BY

COLLINS BUGINGO

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This thesis is approved as a credible and independent investigation by a candidate for the Master of Science degree in Plant Science and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ABBREVIATIONS

ANOVA = Analysis of variance

cm = Centimeter

DAI = Days After Inoculation

DAP = Days After Planting

df = Degrees of freedom

ft = Feet

in = Inches

Kg = Kilogram

LSD = Least Significant Difference

m = Meters

MSDS =Material Safety Data Sheets

NASS = National Agricultural Statistical Services

NERF = North East Research Farm

NGPs = Northern Great Plains

^{o}C = Degree Celsius

P = Probability

Ptr = *Pyrenophora tritici repentis*

SD = South Dakota

SERF = South East Research Farm

spp = species

TPLAD = Total Percentage Leaf Area Diseased

USDA = United States Department of Agriculture

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ABSTRACT

EFFICACY OF SEED TREATMENTS, MICROBIAL AND BIOCHEMICAL PESTICIDES FOR MANAGING EARLY TAN SPOT AND STRIPE RUST DISEASES OF WHEAT

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Wheat is a major cereal crop in the U.S.A and in the world. However, its production is hampered by a number of factors both biotic and abiotic. Foliar diseases like tan spot caused by *Pyrenophora tritici-repentis* and stripe rust caused by *Puccinia striiformis* f. sp. *tritici*, are economically important diseases causing over 5-55 % yield loss. To assess the efficacy of fungicide seed treatments in the management of early tan spot, two hard red spring wheat cultivars "Select" and "Brick" were treated with seed-applied commercially available combo fungicides metalaxyl + pyraclostrobin + triticonazole and difenoconazole + mefenoxam + sedaxane + thiomexotham. A non-treated check consisting of naked seed for both cultivars was included. The treated and non-treated seeds planted in the greenhouse and inoculated with *P. tritici-repenti*s at 7, 14, 21 and 28 days after planting (DAP). Disease severity, lesion size, and number of lesions ratings were taken from inoculated plants at 7 and 14 days after inoculation (DAI) for each inoculation period. To evaluate the efficacy of fungicide seed treatments on stripe rust, two hard red winter wheat cultivars "Expedition" (moderately susceptible) and "Alice" (susceptible) to stripe rust were seed treated with two commercial fungicides pyraclostrobin, and difenoconazole + mefenoxam and planted in cones and then transferred to a growth chamber at 10 ^{o}C. A non-treated seed check was included for

comparison. The plants were inoculated with *Puccinia striiformis* f. sp. *tritici* obtained from a wheat field near Brookings the previous season. Ratings for stripe rust severity were done at 20 days after inoculation for the plants inoculated at 2 weeks after planting. To test the effect of seed treatment on early tan spot development under field conditions, field trials were conducted from the South Dakota State University (SDSU) Northeast Research Farm near South shore in Watertown and at SDSU Volga Research Farm with seed treatments having active ingredients; prothioconazole+ penflufen+ metalaxyl; sedaxane; pyraclostrobin; metalaxyl + ipconazole; difenoconazole + mefenoxam and the untreated seeds as the check. Results from the greenhouse seed treatment control of early tan spot development indicated a significant difference among seed treatments with a low tan spot severity in the treated plants. Untreated plants for both cultivars had the highest percentage disease severity ranging from 50- 70 %, large lesions (0.5-0.6 centimeters) and high lesion counts (30-40) at 1, 2, 3 and 4 weeks after planting compared to the treated pots that had significantly low percentage disease severity, lesion numbers, and lesion size. Similarly, significantly lower tan spot severity was observed in the seed treated plots compared to non-treated in the field trial at both locations. Plots with fungicide seed treatments had higher numbers of plants/m^2 compared to the non-treated. Winter survival was significantly high in the treated plots than the untreated for both locations. Likewise, there was significantly a higher grain yield for plots treated with fungicides than the untreated plots.

To assess the efficacy of the biochemical and microbial pesticides, a hard red spring wheat cultivar “Select” was planted in the cone-tainers. The 3 and 6 weeks old seedlings were pre-treated with products containing active ingredients *Bacillus subtilis*

QST713, *Bacillus amyloquafasciens* D747, *Streptomyces lydicus* WYEC 108, hydrogen peroxide + peroxyacetic acid and azadirachtin. Pyraclostrobin acted as a positive control. All greenhouse plants were then inoculated with *Ptr* three days after initial product application at three and six weeks after planting. The same treatments were used in a field setting and a split-plot design was used for two separate fields, namely; the organic and conventional plots at the Southeast Research Farm. Results from the greenhouse and field study showed significant tan spot disease control in the greenhouse with low tan spot severities in cones treated with pyraclostrobin, *Bacillus subtilis* QST713, *Bacillus amyloquafasciens* D747, *Streptomyces lydicus* Wyec 108. Plant vigor was also significantly increased in terms of greenness with the treated plots having higher green ratings than the untreated. Azadirachtin was not significant at managing the tan spot disease or improving the plant greenness. Likewise, yield was significantly high in plots treated with microbial pesticides than in the control/untreated plots. In a study to establish the sensitivity of *Pyrenphora tritici repentis* to fungicides, ninety isolates collected from North Dakota, South Dakota, Montana, and Kansas were tested for sensitivity using both spore and mycelia assays on picoxystrobin, prothioconazole + tebuconazole and azoxystrobin + propiconazole. From the mycelia assays, thirty out of the ninety isolates tested in microtiter plate assays showed insensitivity to picoxystrobin half and full rates with and without Salicylhydroxamic acid (SHAM). Further assessments of the five *Ptr* isolates involving spores showed germination of all isolates on all fungicides with picoxystrobin having the highest percentage spore germination. The double rate of the fungicides had the least germination percentage for all the isolates tested. Spore germination was nullified when the fungicide picoxystrobin was amended with

Salicylhydroxamic acid (SHAM) and no spore germination was recorded meaning all the isolates were sensitive to the fungicides and the prior registered insensitivity was a result *Ptr* evading the cytochrome *b* site of QoI action in the absence of SHAM. The findings of this study could be an important source of information for growers and the industry in making wheat production more profitable by informed tan spot and stripe rust management decisions.

CHAPTER 1

Literature review

1.1. Domestication and the economic importance of wheat

Wheat *(Triticum aestivum)* is one of the most widely cultivated staple grains globally and is a key source of calories and proteins needed by the human body (Curtis et al. 2002; Breiman and Graur, 1994; FAOSTAT, 2017). However, with the prediction that the world population will exceed 9 billion people by 2050, the production of wheat must be increased to meet the demands (Figueroa et al. 2018). Wheat belongs to the genus *Triticum*, which has 10 species, out of which only 6 are cultivated, and out of the 6, the most cultivated is common wheat, (*Triticum aestivum*). Wheat needs 12 to 15 inches of rain to produce a good crop. Wheat grows best when temperatures are in dimensions of 3°C – 4 °C and 30 °C – 32 °C (Briggle, 1980). The wheat crop also requires low relative humidity in order to escape disease attacks in damp conditions which highly favor infection. Wheat is a highly adaptable cereal that can do well in a wide range of environments as compared to other cereals (Briggle & Curtis, 1987). It is a highly dominant crop in the temperate regions partly because of its good source of gluten protein as well as livestock feed.

Wheat has various uses in the final products it offers like pasta, noodles, bread due to the viscoelastic properties conferred to it by gluten protein. The production of wheat has been embedded in human history, and for over 8000 years it has been a staple food for people in Europe, West Asia, and North Africa. As of today, U.S.A ranks second globally in wheat production and use after the European Union (USDA, 2017).

Cultivation of wheat started about 10,000 years ago (Heun et al. 1997; Dubcovsky & Dvorak, 2007). This is the period man was transitioning from fruit gathering and hunting to settled cultivation. Tetraploid AABB (Emmer) and diploid AA (einkorn) genomes were the first forms of wheat grown, the genetic relationship of einkorn and emmer reveal that they originated from the eastern part of Turkey also known "fertile crescent" (Heun et al. 1997; Dubcovsky & Dvorak, 2007). Carbon dating of the 5th and 6th millennia BC reveals that some wheat grains were found in Northern Iraq in the settlement site of Yarym-Tepe (Bakheyev et al. 980). In the book, "Wheat Breeding" Bell, (1980) points out that wheat cultivation dates way back to 7500-6500 BC. Emmer wheat was later domesticated and reports show it was first grown at the Levant in Eastern Mediterranean (Feldman, 1976).

In the U.S, pure lines dominate wheat production (Koemel et al. 2004) despite efforts to introduce hybrids in the past 28 years. In the Great Plains, three common classes of wheat are grown; hard red spring, hard red winter, and hard white durum (*T. turgidum L. var. durum*), these are used for bread and pasta, respectively. Three cultivars 'Red Fife' hard red spring wheat, 'Turkey'-type hard red winter wheat, and 'Kubanka' durum wheat were bred to adapt to the Great Plain region and this has brought over 16 million hectares under cultivation (Paulsen et al. 2008).

Wheat is of great economic importance both nationally and internationally and the worlds' estimated wheat production for the year 2018/2019 is 747.8 million metric tons- (NASS, 2018). In the USA, wheat ranks third among the U.S field crops and the 2018/2019 projection of wheat to be produced is 49.6 million metric tons (NASS, 2018). USDA reports an annual wheat export revenue of $ 9 billion (USDA, 2017).

1.2 Constraints of wheat production

Wheat is faced with a number of production constraints, both biotic and abiotic. The biotic constraints include but are not limited to pests and diseases. Diseases such as stripe rust, stem rust, leaf rust, Fusarium head blight and tan spot are highly prevalent in the wheat producing regions world over and in the Northern Great Plains (NGP) (Wegulo, 2006; Galich, 1996; Gilbert & Tekauz, 2000; Lori et al. 2003: Mcmullen et al. 1997). Also, Mehta, (2014) cited diseases as a major production constraint to wheat. There are also technological and scientific constraints that affect wheat production, for example, the lengthy process of releasing and adoption of new wheat varieties (Brennan and Byerlee, 1991) and this partly explains the lack of tan spot resistant cultivars in the Great Plains. Stripe rust is one of the most prevalent diseases in the cooler areas of Midwestern (Wiese, 1987). Stripe rust reduces the yield and quality of grain and forage (Chen, 2005). Stripe rust can also cause up to 100% yield losses if the infection is very early and disease keeps increasing all through the growing season (Chen, 2005). It is estimated that stripe rust causes a 2.2-5% yield loss per year (Chai et al. 2014) and the aggressiveness of *Puccinia striiformis var. striiformis* has greatly increased (Milus et al. 2009) which makes it more concerning. There's a paucity of information for the efficacy of seed treatments and organic pesticides in the early management of stripe rust just like for tan spot in the Northern Great Plains (NGPs).

1.3 The economic importance of tan spot disease of wheat

Tan spot is caused by a homothallic ascomycete parasitic fungus *Pyrenophora tritici- repentis (Ptr)* (Died) Shoem.) which is a wheat stubble-borne pathogen (Hosford,

1982). The tan spot fungus survives on wheat stubble (Conway, 1996; Jardine et al. 2000; Krupinsky et al. 2002; McMullen and Lamey, 1994; and Watkins and Boosalis, 1994), a reason it is highly prevalent under no-tillage and where wheat on wheat practice exist. This disease has been named as a disease of man's own making because of farming practices that favor this disease to develop. *P. tritici-repentis* infections can start in the fall in winter wheat especially when there is extended warm fall weather. Thus the use of susceptible cultivars and a shift from conventional tillage to no-till have increased the spread and level of tan spot pathogen inoculum (Hosford, 1982; Lamari et al. 2005; Singh et al. 2007).

Tan spot is an economically important disease because the wheat growing regions of the world are faced with yield losses ranging between 5- 55% (Singh et al. 2010; Shabeer & Bockus, 1988). In South Dakota, 5% grain yield loss has been recorded but individual field grain yield loss can be higher than 30% (Buchneau et al. 1983). Tan spot is still regarded as the most prevalent leaf spot disease in South Dakota (Byamukama, 2013; Friskop & Liu, 2016).

1.4 History and distribution of tan spot disease

Pyrenophora tritici-repentis is a necrotrophic plant fungal pathogen in the phyla Ascomycota (Aboukhaddour et al. 2017). *Pyrenophora tritici-repentis* was first described in 1823 and identified in the USA, Europe, and Japan in the 1900s (Nisikado, 1928; Faris et al.2013). It was isolated from *Agropyron repens*, and earlier described as *Pleospora trichostoma* (Diedicke, 1902), and later in 1903 renamed as *Pyrenophora tritici-repentis* (Shoemaker, 1961 & 1962). This debate of nomenclature kept going on amongst scientists with various names originating from either the asexual or sexual component of

the fungus and this name, *Pyrenophora tritici-repentis* was unanimously agreed upon by Wehyemer, (1954) and Shoemaker, (1961 & 1962).

In Australia tan spot was first reported by Valder and Shaw (1953). A yield loss as much as 29% for trials carried out in Queen's land on four sites was registered (Platz, 1978). In Canada, tan spot was reported in 1974, especially in Western Canada where commercial wheat is mainly grown (Tekauz, 1976). The pathogen *P. tritici-repentis* was isolated from 23 out of 43 fields from Manitoba and Saskatchewan.

Tan spot was first reported in North Dakota in the USA in the 1970s (Hosford, 1971). Tan spot impacted yield a lot to the point that Montana state registered a 19.7% (Sharp et al. 1976). Other states where the disease has been reported with high severity include Kansas, Nebraska, Oklahoma, South Dakota, Mississippi, and Arkansas, among many others. In central and South America, tan spot has been reported in Paraguay, Brazil, Bolivia, Uruguay, Mexico, Argentina (Kohli et al. 1992). Also, it is recorded that tan spot of wheat caused an estimated loss of up to 36% in the state of Parama, Brazil in 1992 (Kohl et al. 1992). A country-wide survey was conducted in Hungary in 1989 and 70% of the area surveyed had tan spot infestation. The same survey was conducted the following year 1990 and there was 51% tan spot infestation (Bokyinia et al. 1998). In Africa, tan spot was identified in triticale in Morocco during varietal evaluations in 1989 – 1990 (El Harrak et al. 1998). Durum wheat was infected with *P. tritici-repentis* in eastern Algeria (Lamari et al. 1989). In Asia, tan spot was found in Bangladesh, China, Thailand, Georgia, Afghanistan, Iran, and Nepal with a recorded yearly loss of 5-10% due to tan spot (Karki, 1982).

1.5 Disease cycle and biology of *Pyrenophora tritici-repentis*

P. tritici repentis mycelia overwinter as black pinhead-sized structures known as psuedothecia which develop on wheat straw (Figure 1). The pseudothecia are described as one-loculed black raised bodies measuring 0.2-0.35 mm in diameter (Hosford, 1972). The fruiting body pseudothecia discharge ascospores in the spring and early summer and the ascospores are the primary sources of inoculum. Ascospores have a limited rate of transmission and can move a short distance of about 6 inches with the help of wind and rain splash (De Wolf et al. 1998). The ascospores possess a cell in the center having 4-5 septa (Ellis & Waller, 1976). The primary infection by the ascospores is favored by damp conditions with temperature ranges between 15 ^{o}C and 28 ^{o}C and the presence of free moisture, this gives 78% and 65% spore germination on susceptible and resistant varieties, respectively, and the percentage rises to 95% after 6 hours for both varieties (Larez et al. 1986; De Wolf and Erick, 2008; De Wolf et al. 1988).

After the establishment of tan spot in the field (2-3 weeks after infection), the plants develop necrotic symptoms. The primary infection gives rise to another set of secondary spores called conidia. The conidia have a transmission rate greater than that of the ascospores hence the conidial spores infect other wheat plants with the help of wind dispersal and rain splash which makes them epidemiologically more important. The conidia are olive-black and possess an inflated base with 4-6 septa with a snake-shaped/tapering head (Figure 2) (Shoemaker, 1962). Secondary infection is favored by leaf wetness, high relative humidity and temperatures above 10 ^{o}C (Hosford et al. 1972; Rees et al. 19779; Sharma et al. 2003).

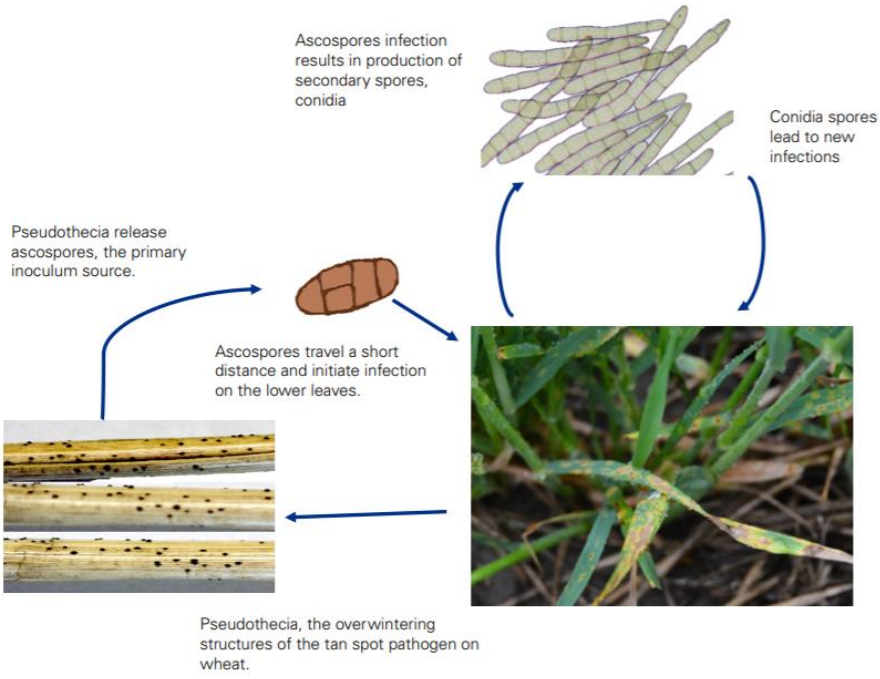

Figure 1.1: Disease cycle of the tan spot

1.5.1 The tan spot fungus infection process

In the presence of favorable weather conditions with temperatures above 10 ^{o}C and a high relative humidity of about 78-100%, the conidia or ascospores produce the germ tube on the leaf which later produces the appressorium from which the penetration peg develops (Hosford et al. 1987). The penetration peg enables the fungus to directly penetrate into the epidermal cells (Larez et al. 1986). The penetration process takes about 3 hours (Dushnicky et al. 1996). After the penetration process, the intracellular vesicle develops from the penetration peg but this is not always the case since the hyphae can penetrate through the epidermis (Larez et al. 1986; Dushnicky et al. 1996). The hyphae extend to the mesophyll layers and the organelles get damaged which consequently develop into the distinctive tan spot symptoms of brown-lens shaped necrotic lesions with a chlorotic halo surrounding it (Figures 4 & 5) (De Wolf et al. 1998; Hosford, 1982).

1.5.2 Symptoms of tan spot disease

Tan spot disease develops both on the lower and upper leaves with necrotic or chlorotic symptoms or both (Lamari and Bernier, 1989a). The tan necrosis and chlorosis symptoms are under independent genetic control because individual isolates *Pyrenophora tritic repentis* induce the symptoms differently (Lamari and Bernier, 1989b). Infected leaves have spots that are tan to brown in color with a lens-oval or diamond shape (Figures 2 & 3). The key diagnostic symptom of tan spot is the "eye shaped" dark spot located in the center of the yellow lesion which distinguishes it from other wheat fungal leaf spotting diseases like Septoria blotch and Stagonospora blotch (De Wolf et al. 1998; Lamari & Bernier, 1989a). The lesions may coalesce and the severely infected leaves finally wilt and die off (De Wolf et al. 1998). Tan spot pathogen infections reduce yield because the photosynthetic area is reduced hence affecting grain quality and quantity due to reduced grain filling, lower test weight, kernel shriveling, and reduced kernel numbers per head, (Shabeer & Bockus, 1988; De Wolf et al. 1998).

Tan spot also causes grain red smudge symptom. This happens during the grain-filling period (Fernandez et al. 1994; Bergstrom & Schilder, 1998). Seeds from infected spikelets have a reddish discoloration symptom (red smudge). The pathogen infects the seed in much the same way as it does for leaves (De Wolf et al. 1998). This means that the disease inoculum can also be seed-borne which explains the introduction of disease where it has not formally been but for the case of the Great Plains, the pathogen is predominantly stubble-borne.

Figure 1.2: Tan spot symptoms (arrow) developing on leaves of wheat planted into wheat stubble.

Figure 1.3: Wheat leaf with tan spot symptoms. The lesions have a dark center (necrotic area) surrounded by the yellow halo (chlorotic area).

1.5.3 Hosts of *Pyrenophora tritici-repentis*

Alternative hosts act as pathogen reservoirs between cropping season and play a vital role in the pathogen reproduction process. They also increase the inoculum for disease development (De Wolf et al. 1998). Tan spot infects other cereals apart from wheat and the major ones are triticale, rye, and barley (Prescot et al. 2014; De Wolf et al. 1998). Wild grass species like bromegrass, sheep fescue, little bluestem, smooth brome, green foxtail, Siberian wheatgrass, June grass, tall wheatgrass, needle and thread (Krupinsky, 1982 & 1992; Hosford, 1971; Ali & Francl, 2003) plus many others are also hosts of *Ptr* and this explains the genetic variability in the tan spot pathogen population (Krupinsky, 1992).

1.6 Management of tan spot

Tan spot is a devastating disease causing yield loss ranging from 5-55% (De Wolf & Hoffmann, 1993; Shabeer & Bockus, 1988; Buchneau et al. 1983; Singh et al. 2010). Also, most of the cultivars available commercially in the Great Plains are susceptible to moderately susceptible to tan spot. Tan spot is more severe and highly prevalent in the fields where no-till and wheat on wheat is practiced as farmers aim at conserving soil

moisture and control soil erosion (Carigano et al. 2008; Ronis et al. 2009). The management of tan spot encompasses; stubble management, fungicide application, use of resistant cultivars and crop rotations.

Since *Pyrenophora tritici repentis* survives on wheat stubble, practices such as crop rotations and tillage where practical will starve the pathogen and hence break its cycle. Summerel and Burgess, (1989) established that burial of infected stubble was more effective in controlling tan spot than the use of a rotary hoe. However, this comes with its own limitations like disturbing of the soil biodiversity and risking to soil erosion.

Research shows that reduced or no-tillage has increased the prevalence and spread of tan spot (Wiese, 1987, Schuh, 1990). Related studies also revealed a significant relationship between tan spot disease severity and the primary inoculum load (Adee et al. 1989). Crop rotation is considered the most effective control measure for long-term benefits (Bockus et al. 1992) and the non-host crops to be considered for a rotation include mustard, soybean, corn, and flax (Bockus & Claasen, 1992).

Adequate but not excessive fertilization was found to reduce the disease complexity of tan spot in fields under no-till cultivation practices in the Northern Great Plains (Wiese,1987; Krupinsky et al. 2007). In Indiana, Huber et al (1987) reported a decrease in tan spot on soft red winter wheat cultivars as the nitrogen rate increase. Related studies in Saskatchewan attributed greater Septoria/Stagonospora development on winter wheat to low nitrogen (Tompkins et al. 1993).

Planting of resistant varieties is the most effective and durable way of managing tan spot. Breeding programs endeavor to develop disease-resistant cultivars in order to have sustainable wheat disease control. Durum and bread wheat varieties that are resistant to

the tan spot pathogen are available. *Ptr* produces host-selective toxins (HSTs) and these are important when breeding for tan spot resistance. Host-selective toxins (HSTs) produced by fungal plant pathogens are generally low-molecular-weight secondary metabolites with a diverse range of structures that function as effectors controlling pathogenicity or virulence in certain plant-pathogen interactions. The toxins in *Ptr* are Ptr ToxA, Ptr ToxB, and Ptr ToxC (Sensu Yoder, 1980). The toxins are matched to a corresponding resistant gene in the host plant sn1, Tsc2, and Tsc1, respectively. Tan spot has been described as the only pathogen that can produce multiple HSTs that differentially allow compatibility on differential lines of a single host species (Lamari et al. 2003). Resistance Quantitative Trait Loci (QTL) and recessively inherited qualitative resistance genes have been discovered (Faris et al. 2013). A QTL is a locus which correlates with a variation of a quantitative trait in the phenotype of a population of organisms (Miles and Wayne, 2008). Molecular markers suitable for marker-assisted selection against HST sensitivity genes and for race non-specific resistance QTLs are being used to generate adapted germplasm with quite good levels of tan spot disease resistance (Faris et al. 2013). Some key sources of tan spot resistance are the wheat wild relatives, synthetic hexaploid wheat lines and tetraploid wheat relatives among others (Chu et al. 2008). Unfortunately, we do not have any tan spot resistant cultivar in the Dakotas as of now (Kleinjan, 2015).

Use of fungicides in the class strobilurin and triazole have proved effective in the management of tan spot. Bockus et al (1992) and Loughman et al (1998) found an increase of 21-43% of large seed when a fungicide is applied to susceptible cultivars. Tebuconazole, a systemic fungicide has been reported effective in the management of

numerous wheat foliar disease (Anonymous, 2015; Poole & Arnaudin, 2014). A study by Turkington et al (2016) revealed successful management of leaf spotting diseases including tan spot when a combination of foliar and seed fungicide treatments was applied in the Northern Great Plains. Fungicides applied in the early stages of crop growth do not provide direct protection to the upper canopy (Poole & Arnaudin, 2014; Turkington et al. 2004, 2015) yet the flag leaf is so critical in the maintenance of seed yield. However, the excessive use of fungicides might end up creating a selection pressure which can result in fungicide resistance, and this has been documented in some countries like Germany, France, Sweden, and Denmark (Sierotzki et al. 2007).

A number of biocontrol organisms were found to be effective on the tan spot fungus, these *are Alternaria alternata, Fusarium pallidoroseum, Acinetobacter calcoaceticus, Serratia liquefaciens, Limonomyces roseipellis, Paenibacilluss maceruns* and *Trichoderma harzianum* (Gough & Ghazanfari, 1982, 1982; Luz et al. 1998). These can either reduce the pathogen competitiveness, stop or limit its growth and sporulation or induce systemic resistance in the host plant (Benhamou et al. 2001).

1.7 Stripe rust of wheat

1.7.1 History of stripe rust in wheat

Stripe rust, also known as yellow rust is another important disease of wheat (Hovmøller et al. 2011; Wellings, 2011). This rust is caused by a biotrophic fungus *Puccinia striiformis* Westend. f. sp. *tritici Erikss*. Stripe rust pathogen variability is driven by mutation, somatic recombination, selection, parasexuality and sexual recombination (Duan et al. 2010; Hovmøller et al. 2011; Mboup et al. 2009; Stubbs,

1985). References to the epidemics of stripe rust date way back to the Old Testament Biblical writings by Moses and the writings of early scholars (Large, 1940). There are speculations that the wheat disease epidemics reported in England in 1725 and Sweden in 1794 may have been due to stripe rust (Chester, 1946). The center of origin for *P.striiformis* is assumed to be the Transcaucasia, where it moved into Europe and along the mountain ranges from China and Eastern Asia entering America through the Aleutian Islands and Alaska and infected the native wild grasses (Stubss, 1985; Humphrey et al. 1924). *P.striiformis* pathogen was first described in Europe by Gadd and Schmidt in 1777 and 1827 (Eriksson and Henning, 1896; Humphrey et al. 1924; Stubbs, 1985). Eriksson and Henning are said to have named the fungus *Puccinia glumarum* based on its telial stage (Humphrey et al. 1916). Scientists Hassebrauk (1965), Stubbs (1985), Line (2002), and Li and Zeng (2003) were the first to report the global distribution of stripe rust. It has been reported in more than 60 countries on all continents with an exception for Antarctica (Chen, 2005). In the USA, stripe rust was first discovered in 1915 by F. Kolpin Ravn, who was traveling with a United States Department of Agriculture (USDA) crop survey team in the western United States (Humphrey, 1917; Carleton, 1915; Humphrey et al. 1916 & 1924). However, retrospective historical reviews have indicated that stripe rust had been in the USA twenty-three years before its identification in Oregon, Washington, Montana, and Utah (Humphrey et al. 1916 & 1924; Carleton, 1915).

1.1.1 The economic importance of stripe rust

Stripe rust is a serious disease of wheat occurring in most wheat growing areas with moist and cool weather conditions (Hovmøller et al. 2011; Wellings, 2011). In the U.S, Stripe rust is said to be more spread in the Western USA (Chen, 2007). Stripe rust in

South Dakota's winter wheat is detected towards the end of May / early July (Byamukama, 2016). South Dakota did not register any stripe rust in 2018 unlike 2017 (Byamukama et al. 2018) but this does not mean that farmers should relax since the disease is dependent on the factors of a favorable environment, a susceptible host and a virulent rust spore. All these can be controlled and predicted to a limited extent a reason management practices like use of fungicides, planting resistant cultivars, and use of prediction tools have to be used as an IPM system to curb down the economic losses that arise from stripe rust.

Stripe rust was found to be the most severe and a significant threat in the wheat growing regions and the regular regional crop losses are estimated between 1-5% with some peculiar small cases of between 5-25% losses (Welling, 2011). Yield losses as high as 65% have been recorded in the greenhouse studies using susceptible cultivars (Bever, 1937). Wheat cultivar PS 279 which is stripe rust susceptible has registered 100% yield loss (Chen, 2005). In other studies, stripe rust is said to cause grain yield losses of up to 84%, a reduction in kernel mass of 43% and a decrease in the kernel number of 72% (Murray et al. 1995). Noteworthy was the 2.9 million-bushel and 7.3% loss that was recorded only in the state of Washington and Kansas in the year 1958 and 2000 respectively (Chen, 2005). Numerous other yield losses have been recorded in the states of Idaho, Colorado, Missouri, Nebraska, Louisiana, Oregon, and many others. In the year 2003, the great plains recorded a huge loss of (2.42×10^6) and this is attributed to the large acreages of susceptible wheat grown (Chen, 2004).

1.7.2 Symptoms of stripe rust

Puccinia striiformis (*Pst*) infects the green tissues of cereals and grasses. As long as the plants are still green and the conditions are favorable, the infection will occur at any stage of plant growth right from one leaf stage (Chen, 2005). Stripe infection occurs from 0-25 ^{o}C with free water on the leaf (Stubbs, 1985) and this will initiate symptoms in one week after infection and thereafter sporulation starts at about 2 weeks after infection. *Pst* presents itself with tiny, yellow-orange-colored pustule (uredia). In each uredium are thousands of microscopic urediospores and an individual urediospore is too tiny to be seen with a naked eye. However, the stripe rust spores can be seen in a mass of yellow to orange color and are powdery in form and loosely attached on the green tissues of the host. The stripes of the uredinia (elongated spots) will not form until after stem elongation hence are not visible at the seedling stage (Chen, 2005). There are varying amounts of necrosis and chlorosis caused by stripe rust pathogen and these depend on the temperature and the host's level of resistance.

1.7.3 Life cycle of *Puccinia Striiformis*

Wheat rust pathogens like *Puccinia striiformis*, are known for their rapid and widespread because of their ability to produce large volumes of spores. It is estimated that rust pustules can produce 10,000 urediospores per day and each will, in theory, produce a new pustule within 10 days. The life cycle of stripe rust is complex, involving five different spore stages including the asexual teliospores (2n) basidiospores (n), and involving the sexual spores pycniospore (n) and aeciospores (n+n) on the alternate host Barberry which finally produces the uredinia that releases urediospores (n+n) on wheat (Figure 1.4) (Jin et al. 2010; Wang and Chen, 2013; Zhao et al. 2011 & 2013). Stripe rust

is regarded as a low- temperature disease appearing mostly in the cool, wet seasons. The optimal spore germination is 10-12 ^{o}C with free moisture on the leaves whereas for disease development it requires a temperature range of 12.7 -15 ^{o}C (Chen, 2005). Noteworthy is that in most other wheat growing regions, the urediospores are regarded as the only inoculum for initial and continuing infection which makes it the mini stripe rust cycle. The role of the sexual reproduction phase from the recently identified barberry host in genetic variation and race evolution of *Pst* remains a mystery (Zheng et al. 2013). The produced urediospores are powdery and light which makes them easy to be wind-blown to other plants hence initiating new infections over hundreds or thousands of miles. In North America, the spores migrate from Northern Mexico and South Texas through the Northern Great Plains and other states.

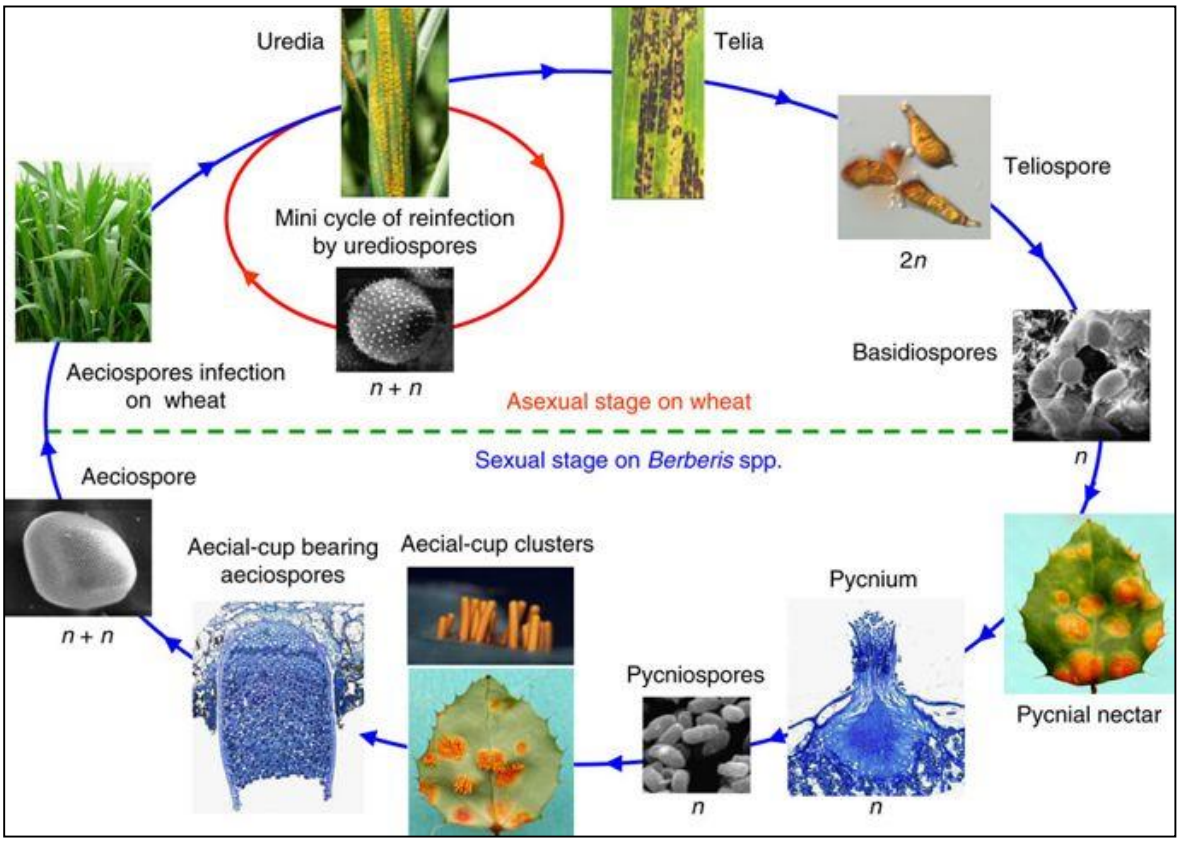

Figure 1.4: Life cycle of *Puccinia striiformis*. Diagram courtesy of Zheng et al. 2013

1.8 Management of stripe rust in wheat

Stripe rust is best managed by growing resistant cultivars (Chen, 2007). An approach of combining genes that are individually effective against stripe rust will most likely offer long-lasting resistance to the host plants (Chen, 2007). The use of High-Temperature Adult Plant (HTAP) resistance has proved successful in the development of stripe rust resistant cultivars (Chen, 2007) especially in the Northern Great Plains and North West USA pacific wheat cultivars. However, HTAP being controlled by quantitative trait loci (Milus & Line 1986; Chen & Line, 1995a & 1995b) has downfalls of difficulty to incorporate in commercial cultivars unlike for the single-gene controlled all-stage resistance. Another limitation of the HTAP resistance is for scenarios where disease keeps developing even under winter or low temperatures and affecting seedlings. This is because HTAP is limited to adult plants and high temperatures. Hence the best mode of resistance with durability and efficacy for the USA is the use of HTAP and all-stage resistance (Chen, 2007).

Use of chemical fungicides is also successful in the control of stripe rust (Boshoff, 2003; Chen, 2007; Byamukama, 2015). In some parts of the US, chemical control of stripe rust was not tried until the 1950s and 1960s (Chen, 2007). Fungicide seed treatments like triadimenol and tebuconazole have proved to be effective in the suppression of stripe rust (Rakotondradona & Line, 1984; Scott & Line, 1985; Boshoff et al. 2003). Foliar fungicides like Tilt (propiconazole), Quadrisr (azoxystrobin), Strategor (propiconazole + trifloxystrobin), Headline (pyraclostrobin), and Quilt (azoxystrobin + propiconazole) are also effective at controlling stripe rust if applied per the recommended guidelines (Chen, 2007). Like other reported fungicide-resistant pathogens, *P. striifomis*

is no exception. Rust fungi are classified as a low risk in terms of fungicide resistance because they are dikaryotic hence differentially express genes from each nucleus in the dikaryon but this might not be true for all fungicides and rust fungi (Oliver, 2014). This is because other dikrayotic pathogens such as *Ustilago*, *Plasmopora*, and *Phytophthora*, are all classified in the medium to the high class of resistance and many resistance scenarios have been registered. This means that growers cannot rely on the dikaryotic nature of rusts to rule out the possibility of fungicide resistance (Leroux & Berthier, 1988). That said, it is encouraged to alternate chemistries while applying fungicides against stripe rust disease and not depend on the claims of limited *Pst* fungicide resistance from long ago. Also, these claims are based on premises of limited *Pst* exposure to fungicides (Scherm et al. 2009; Murray & Brennan, 2010).

Other stripe rust management approaches are cultural for example; late planting, reduced irrigation, avoiding excess nitrogen use, elimination of grass and wheat volunteer hosts will all reduce the severities of stripe rust. However, these practices are conflicting with conservation farming, are non-profitable, or reduce yield potential and all these make breeding for cultivars with commendable resistance the best approach to control this ruinous stripe rust disease (Chen, 2007).

1.9 Use of biopesticides and other organic substances in the management of tan spot in wheat

1.9.1 Background to biopesticides in plant disease management

Use of fungicides coupled with resistant varieties, irrigation, mechanization, and use of fertilizers have increased yields to almost 100 % in the US and 70% in Europe (Pretty,

2008). However, this might not be true as of now due to a number of issues related to the use of synthetic fungicides (Romeis & Meissle, 2006). There's a big shift to the use organic and biopesticides. Biopesticides are certain types of pesticides derived from natural materials like animals, plants, bacteria, and certain minerals, for example, canola oil and baking soda have detrimental effects on pesticides and hence are and are considered biopesticides (EPA, 2018). Biological control can also be defined as an "environmentally-friendly" strategy using living microorganisms or their derivatives to reduce a targeted pathogen population (Flint & Dreistadt, 1998). Part of the biological control approach is the use of biocontrol agents (BCA) whose narrow spectrum provide an optimum efficacy without affecting non-targeted organisms (Alabouvette et al. 2006). Plant extracts were likely the earliest agricultural biocontrols, as history records that nicotine was used to control plum beetles as early as the 17th century (BPIA, 2017). This is because the inconsiderate use of pesticides without taking in contemplation the set safety and recommended-use guidelines has birthed a number of health and environmental risks to both humans and other living organisms (Damalas, 2009; Carvalho, 2017). This explains the growing demand for food safety and quality in the recent years which has also been reflected in the stringent import and export regulations especially on the minimum acceptable amount of residues on commodities (Damalas, 2018).

Every year there is an increase in the usage of biopesticides world over (Marketsandmarkets.com, 2018). This is because the continued use of synthetic pesticides presents a number of challenges like pest resurgence due to a selection pressure created by erratic use of synthetic chemicals and other environmental and

consumer health-related concerns. The use of synthetic pesticides also presents higher likelihoods of eliminating beneficial organisms (Hassan et al. 2014) which ultimately destabilizes the ecosystem (Schuler et al. 1998). Chemical control measures are also ineffective against some of the recorded worst pests, for example, rice brown planthopper (*Nilaparvata lugens*) that feeds by sucking sap from the phloem (Romeis & Meissle, 2006). On the other hand, biopesticides are gaining enormous popularity because of the sustainability benefits and the increasing concerns of the impact of residues arising from the overuse of the synthetic chemical pesticides (Pertot et al. 2015). The biopesticides are far less toxic with no to less residual effects as compared to the conventional pesticides hence less pesticide pollution problems associated with them. They (biopesticides) also degrade faster and are not persistent in the environment. It is however advised that biopesticides be used as a part of the Integrated Pest Management (IPM) program, not as "stand-alone" (Leahy et al. 2014). Use of agents like *Bacillus, Streptomyces* and *Lysobacter* that can produce effective lytic enzymes or antibiotics confer protection to the plants against tan spot (Luz et al. 2003; Whipps, 2001; Zhang et al. 2001) and these are already marketed commercially (Fravel, 2005). Studies of using biopesticides like *trichoderma* which effectively inhibits *Ptr's* saprophytic growth in the residue (Fernandez, 1992; Gilbert & Fernando, 2004) proved to be successful in tan spot management. Several other bacterial and fungal species exhibit antagonistic properties against wheat pathogens like *F. graminearum,* (Legrand et al. 2017; Bujold & Paulitz, 2001).

In fear of the interceptions, many export, import companies, and individual growers are resorting to organic products which not only have low residues and easily

broken down but also fetch highly on the international and local markets. By 2007, there were 1400 biopesticides in sell and now the number has gone high (Marrone, 2008). In 2011, there were 68 and 202 registered biopesticides active ingredients in the EU and USA respectively (Chandler et al. 2011). Currently, the biopesticides comprise $ 3 billion which accounts for 5% of the total crop protection market (Damalas, 2018). The value of the biopesticides market is expected to skyrocket to USD 6.60 billion by 2022, from USD 3.22 billion in 2017, at a Compound Annual Growth Rate (CAGR) of 15.43% during the forecast period (MarketandMarkets.Com, 2018). This anticipated growth is so huge and should benefit growers and the producers in return. The recent strains of the fungus *Talaromyces flavus* SAY-Y-94-01, extracts of the plant *Clitoria ternatea* (butterfly pea), products of the fungus *Trichoderma harzianum*, *Bacillus thuringiensis* var. *tenebrionis* strain Xd3 (Btt-Xd3) and others have proved to possess some potential fungicidal activity but there's a need for field research to make such conclusions based on a more diverse environmental set up and also varying cropping systems (Damalas, 2018).

1.9.2 Biopesticides and other organic substrates in the management of wheat foliar diseases

Biopesticide is abroad term that encompasses many aspects. These include; Microbial (viral, bacterial and fungal) organisms, entomophagous nematodes, plant-derived pesticides (botanicals), secondary metabolites from micro-organisms (antibiotics), Insect pheromones applied for mating disruption, monitoring or lure-and-kill strategies, genes used to transform crops to express resistance to insect, fungal and viral attacks or to render them tolerant of herbicide application. However, for this paper,

three major biopesticides falling into three classifications will be discussed. These are; Biochemical, Microbial, and Plant-Incorporated Protectants (PIPs).

1.9.3 The biochemical pesticides

Biochemical pesticides are naturally occurring substances. They can also be synthetically derived equivalents that have a non-toxic mode of action to the target pests and have been exposed to humans and animals but have minimal toxicity (Leahy et.al. 2014). Examples of biochemical products are, natural plant and insect regulators, naturally occurring repellents and attractants, semiochemicals (insect pheromones and kairomones), induced resistance promoters, and enzymes. Most biochemical pesticides, except for pheromones, are species-specific and are broader spectrum pesticides than the microbial pesticides. They also may have lethal effects upon the target pest. Lethal but non-toxic biochemical pesticides include suffocating agents (e.g. soybean oil), desiccants (e.g. Acetic acid), and abrasives (e.g. diatomaceous earth).

1.9.4 Microbial pesticides

These are microorganisms that produce a pesticidal effect. They have modes of action that often include competition or inhibition, toxicity and even use of the target pest as a growth substrate (Leahy et al. 2014). Microbial pesticides can control many different kinds of pests although each separate active ingredient is relatively specific for its target pests. For example, there are fungi that control certain weeds and other fungi that kill specific insects. The most widely used microbial pesticides are subspecies and strains of *Bacillus thuringiensis* (*Bt*). The strains of *Bt* produce different mixes of proteins/cry proteins (the *Bt* d-endotoxin) and each of these kills a specific pathogen or pathogens of the related species. *Bt* endotoxin is capable of causing lysis of gut cells when consumed

by a susceptible insect (Gill et al. 1992). It should be noted that almost 90% of the microbial pesticides available on the market are derived from one entomopathogenic bacterium; *Bacillus thuringiensis* (Kumar & Singh, 2015).

Microbial pesticides include; Prokaryotic microorganisms like the bacteria eukaryotic microorganisms for example, protozoa, algae and fungi like *Coniothyrium minitans* which is a naturally occurring fungus that has been commercialized to control sclerotinia plant diseases through parasitism of the resting structures of the pathogen, *Paecilomyces fumosoroseus* and *Paecilomyces lilacinus* are used to control root insect pests and nematodes (BPIA, 2017). And finally, the autonomous replicating microscopic elements, including, but not limited to, viruses. Other examples of microbial pesticides are *Blasticidin*, which was first isolated from the soil actinomycete *Streptomyces griseochromogenes* in 1955 by Takeuchi et al and is a contact fungicide with protective and curative action and exhibits a wide range of inhibitory activity on the growth of bacterial and fungal cells with a mode of action mode that the inhibits protein biosynthesis by binding to the 50S ribosome in prokaryotes at the site of gougerotin and this leads to the inhibition of peptidyl transfer and protein chain elongation (Haung et al. 1964). Other earlier used biopesticides are Kasugamycin which was isolated from the soil actinomycete *Streptomyces kasugaensis* Umezawa and first described by Umezawa et al in 1965, *Mildiomycin* produced by the soil actinomycete *Streptoverticillium rimofaciens* strain B-98891(Harada & Kishi, 1978) and is active against powdery mildew (Om et al. 1984); *Natamycin,* a secondary metabolite of the actinomycetes *Streptomyces natalensis* and *S chattanoogensis*, is also known aspimaricin and tennectin, and controls

fungal diseases, in particular, basal rots caused by Fusarium oxysporum in bulbs such as daffodils (Struyk et al . 1958).

1.9.5 Plant-Incorporated-Protectants (PIPs)

United States Environmental Protection Agency (US-EPA) defines plant-incorporated protectants as pesticidal substances produced by plants and the genetic material necessary for the plant to produce the substance. The best example here is the *Bt* pesticidal protein that is introduced into the plant's genetic material upon which the plant manufactures the pesticidal protein which controls the pest when it feeds on the plant (EPA, 2018). PIPs are also grouped in Genetically Modified Organisms (GMOs).

1.10 Fungicide seed treatments for plant disease management

1.10.1 Importance of Fungicides in U.S crop production

Plant pathogenic fungi are so numerous and ubiquitous (Regsdale et al. 1991). The fungi require a host upon which they release countless spores and withdraw nutrients from an infected plant (Gainessi & Reigner, 2006). These fungi affect the quality and yield of crops. This calls for management approaches like the use of fungicides and resistant cultivars. For example, in 1953 it was reported that synthetic fungicides were used on 75% of U.S. potato acreage (Brandes, 1953). The research on the use of fungicides began in the 1940s and this led to increased yields due to improved diseases control (Gainessi & Reigner, 2006). Some of the historical incidences are Bordeaux mixture which reduced cranberry rots by 50%, sulfur applications to peaches in Georgia reduced brown rot losses to 13% (Jones et al. 1912). USDA reports on strawberries, carrots, and fresh tomatoes indicated that national production of the three crops would

decline by 58%, 24%, and 60% respectively without fungicides (Davis et al. 1999; Davis et al. 1998; Sorenson et al. 1997). USDA has also reported negative state specific losses where no fungicides were used for example Michigan potato yield –50%; Georgia sweet corn yield –40%; Florida pepper yield -100%; Massachusetts tomato yield –75%; Washington asparagus yield –60%; California spinach yield –40%, Florida citrus –50%; Maine potatoes –100%; California grapes –97%; Michigan apples –100%; California lettuce –47%, and Texas onion –60% (Knutson et al. 1993; Davis, 1991; Johnston, 1991). Fungicides are important in improving crop vigor, quality, and yield (Turkington et al. 2016; Mathre, 2001; Gianessi & Reigner, 2006; Lucas et al. 2015; Sharma- Purdue et al. 2016; Menzies and Gilbert, 2003; Wiese, 1987).

1.10.2 History of fungicide seed treatments

The history of seed treatments rates way back to over 300 years (Fischer and Holton, 1957; Neergaard, 1977). In 1170 there was a ship carrying wheat grains and it went down off the coast of England near the city of Bristol. This ship was near the coast in that some farmers were able to recover some of the wheat grain. The fact that this grain had been water soaked it was deemed unfit for flour processing and hence farmers opted to plant the seeds. To the surprise of every one the seed that had been soaked in seawater was free of smut as compared to the seed that had not been soaked in seawater which had a heavy smut infestation. All this occurred long before Tillet (1755) had established that the seed borne-fungi (*Tilletia tritici*, *T. laevis*) caused bunt of wheat and that it could be controlled by seed treatments of lime, or lime and salt. In the 16th century, (Woolmann & Humphrey, 1924; Buttress & Dennis, 1959) discovered that common bunt was controlled by soaking seed into salty water. Various techniques

of controlling smut using salt, lye, urine were tried out and in 1770. A French botanist Tillet published an article that highlighted the significant control of smut by such materials. In 1807, a Swiss scientist, Prevost illustrated that treating smut spores with copper sulfate solution inhibited their germination but its use was stopped because it is a general biocide and injures seed germination. In 1912, organic mercury compounds were found effective against the common bunt. These were initially too expensive and had to be applied as liquids which left seeds wet hence hard to use without drying. Further research on mercury led to the first discovery of a commercial formulation Panogen (methylmercury guanidine). Mercury products also presented threats to human and animal health because of their toxicity and this sealed the fate of organic mercury products. This triggered research on copper carbonate which was found safer than copper sulfate and could be used as a powder formulation. Copper carbonate was first used in Australia and later in America (Mathre, 2001). Other seed treatments used in history include hexachlorobenzene against common bunt in the Pacific Northwest (Purdy, 1965) and hot water (Maude, 1996). All the above seed treatment had shortcomings and later carboxin, the first systemic fungicide and its efficacy were observed on loose smut which is a disease that can survive from one season to the next inside the seed. Carboxin was able to halt hyphae growth inside the infected seed. bean Benomyl, captan and diazinon combination (Maude & Kyle, 1971) also managed seed-borne anthracnose when applied as seed treatments. There was a good post emergency activity with indar (fenbuconazole) against wheat brown rust (*Puccinia recondita*) (Rowell, 1976). In Washington, 100% control of yellow rust in winter wheat at the booting stage was registered using seeds treated with triadimefon (Rowell, 1976).

Likewise, ethirimol (C11H19N3O) demonstrated good control of powdery mildew in cereals (Marsh, 1977). Edgington (1980) asserts that there's a potential control for airborne diseases like powdery mildews using soil drenches with dimethirimol, benomyl, or carbendazim. Today we have hundreds of fungicides used for seed treatments both the organic and the synthetic.

1.10.3 Mode of action of fungicide seed treatments

Different seed treatments have different chemistries and modes of action (Mathre, 2001). The first discovered fungicide seed treatment carboxin inhibits mitochondrial function by disrupting the tricarboxcylic acid cycle (TCA) which ultimately hampers with the respiration of the pathogen. Carboxin acts on the complex II of succinate dehydrogenase enzyme (Sierotzki & Scalliet,2013). Fungicides in the Methyl benzimidazole-2-yl Carbamate (MBC) class like thiabendozole bind to the protein tubulin and ultimately arrests nuclear division by interfering with microtubule assembly (Davidse, 1975; Howard et al. 1977). Azoles are the latest fungicides on market and these inhibit demethylation position 14 of lanosterol or 24-methylene dihydrolanosterol (Buchenauer,1987). Another mode of action by fungicides is by limiting the accumulation and incorporation of glucose and mannose into hyphal wall membrane glucans. This interferes with membrane-bound transport processes like phosphorylation of glucose (Lyr,1995). These fungicides are called benzodioxoles, for example, a seed treatment fungicide fludioxonil. Metalaxyl, another seed treatment fungicide affects only the oomycetes by inhibiting the incorporation of uridine into RNA. The turnover of ribosomal RNA deprives the cell of its ribosomes leading to decreased protein synthesis (lyr, 1995).

1.11 Fungicide resistance in *Pyrenophora tritici repentis*

Fungicides achieve efficacy through interference with fungal cellular processes which ultimately halt growth. Fungicide Resistance Action Committee (FRAC) defines fungicide resistance as an acquired, heritable reduction in sensitivity of a fungus to a specific anti-fungal agent (FRAC, 2018). Fungicide pathogen resistance is becoming widespread and as a result, the performance of most modern fungicides has been affected to a certain degree (Keith & Holloman, 2007). This is partly because of mutations arising from the use of fungicides with the same chemistries. Fungicide resistance can be quantitative or qualitative. Quantitative resistance is polygenic (Brent & Hollomon, 1998). This affects several fungicides with different modes of action. Quantitative resistance is a result of inadequate intracellular fungicide concentrations by enzymatic degradation of antifungal compounds, fungicide secretion by plasma membrane-localized efflux transporters (Del Sorbo et al. 2000) and utilization of alternative metabolic pathways. Qualitative resistance arises from mutations in genes encoding fungicide targets (Ishi et al. 2001; Ma et al. 2003)

Fungicide resistance to the systemic fungicides is said to have had a rapid evolution in some populations of plant pathogenic fungi. Peever & Milgroom (1992) and Metcalfe et al (2000) demonstrated that doses that provide the most control of diseases are at a higher risk of resistance because they create a selection pressure. In the same study, Metcalfe et al indicated that lower doses decrease the selection pressure. The evolution and process of fungicide resistance are in two phases i.e. the emergence and selection phase (Van den Bosch & Gilligan, 2008; Miligroom, 1990; Van den Bosch et

al. 2011). In the emergence phase, the fungicide-resistant strain comes from mutation. In the selection phase, it is the evolved resistant strain from a mutation that increases in frequency due to the application of fungicides killing the rest of the population (Van den Bosch & Gilligan, 2008 & Van den Bosch et al. 2011). The selection phases are our very concern because as growers keep using fungicides routinely there are higher risks of selection pressure (van den Bosch et al. 2014). It, therefore, should be noted that repeated use of fungicides with the same mode of action can result in the selection of fungicide-resistant strains of plant pathogens that were initially sensitive to the applied fungicide.

1.12 Mode of action of major fungicide classes upon which resistance is based

Fungicides achieve their efficacy through a mode of action (MOA). A mode of action refers to the biochemical pathway being targeted within the pathogen (FRAC, 2018) or the specific cellular process that is inhibited by a particular fungicide. There are different letters (A to I, with added numbers) that are used to put a distinction between fungicide groups according to their biochemical mode of action (MOA) in the biosynthetic pathways of plant pathogens (FRAC, 2018). This grouping is based on the processes in the metabolism right from the nucleic acid synthesis (A) up to secondary metabolism (FRAC, 2018). Examples of secondary metabolism are melanin synthesis (I), host-plant inducers (P), molecules with unknown mode of action and unknown resistance (U) and the chemical multi-site inhibitors (M). The biological pesticides are grouped according to their main mode of action in the respective pathway categories (Appendix 1). A newly introduced category “Biologicals with multiple modes of action” (BM) is used for agents from biological origin showing multiple mechanisms of action without

the evidence of a dominating mode of action (FRAC, 2018). Fungicides affecting the same target site within a biochemical pathway are said to have the same mode of action whereas those affecting different sites are said to have multiple modes of action. Fungal pathogens develop resistance (insensitivity to fungicide applied) to a mode of action hence rendering the active substance less effective and this is a result of the evolution of a mutation(s) in the target pathogen's genome. Fungicides that target single protein binding sites are called single site systemic fungicides whereas the ones that target multiple cellular processes are called multi-site target fungicides (Lucas et al. 2015). The single-site fungicides which include the modern selective fungicides are highly prone to resistance than the multi-site target fungicides (Grimmer et al. 2014).

Triazoles are widely used and this dates to way back in the 1980s. They have a broad spectrum of plant pathogens on which they exert their effect. The mode of action of triazoles is by inhibiting the cytochrome P450 sterol 14α-demethylase (CYP51) enzyme that performs the role of sterol biosynthesis (Buchenauer, 1987). When the enzyme is inhibited, the fungal membrane integrity is compromised. Ergosterol, the 4α-demethylated product are ubiquitous components of the plasma membranes and play an important role structurally to regulate membrane fluidity and permeability. Ergosterol also indirectly modulates the activity and distribution of integral membrane proteins, including enzymes, ion channels and components of signal transduction pathways (Nes et al. 1977; Schaller, 2003). The triazole fungicides are also termed as the demethylation inhibitors (DMIs) (Kuck et al. 1995). Other examples of DMIs include pyrimidine, piperazines, and pyridine fungicides. In contrast to other site-specific fungicides that have been rendered ineffective as a result of point mutations at the genes encoding target

proteins, the DMIs have a decreased and gradual loss of efficacy (Brent & Holloman, 2007). Fungicide resistance in azole fungicides is attributed to the target-site alterations based on point mutations in the CYP51 gene (Wyand et al. 2005; Leroux et al. 2007), changes in the sterol biosynthesis and sterol composition (Joseph-Horne et al.1996; Joseph-Horne et al.1995), overexpression of the target gene (Schnabel et al. 2001; Hamamoto et al. 2000), enhanced energy-dependent efflux transport of the toxic compounds (Nakaune et al. 2008), additive effects (Wellmann et al. 992; Selmecki et al. 2008; Joseph-Horne et al. 1995) and increased copy numbers of the target gene and the transcriptional regulator of the drug efflux pumps (Selmecki et al. 2008).

The Quinone out Inhibitors (QoI) also known as strobilurins suppress a wide range of diseases including those caused by water molds, downy mildew, powdery mildews, leaf spotting and blighting fungi, fruit rotters, and rusts. The QoI class of fungicides interferes with one or more of the biochemical pathways hence hampering pathogen growth. The QoI affect mitochondria respiration which ultimately shuts down the energy source for the pathogen. The QoI's target site of action is the complex 111, cytochrome bc_1 (ubiquinol oxidase) at Qo site (Cyt b gene). Cytochrome b is a part of the bc_1 complex in the inner mitochondrial membrane of the fungi and other eukaryotes and it achieves its function by blocking the electron transfer between cytochrome b and cytochrome c_1 after one of the inhibitors have bound on it (Barlett et al. 2002). This ultimately tampers with the energy cycle within the organism by the stoppage of ATP production. Q_oI fungicides act excellently as preventives, not curatives, mainly because they all effectively kill germinating spores (Vincel, 2002). Examples of QoI are methoxy-

acetamide, methoxy-carbamates, oximino-acetates, oxazolidine-dones, dihydro-dioxazines, imidazolinones, and benzyl-carbamates.

Succinate Dehydrogenase Inhibitors (SDHIs) are FRAC group 7 that targets the complex 11 or succinate dehydrogenase enzyme. The examples in this group include Phenyl-benzamides, phenyl-oxo-ethyl thiophene amide, Pyridinyl-ethyl-benzamide, Furan-carboxamides, Oxathiin-carboxamides, Thiazole-carboxamides, Pyrazole-carboxamides, Pyridine-carboxamides (SDHI working group). Fungal resistance to SDHIs exist and this varies between pathogen species, SDHIs used and the geographic location of the isolates (Sierotzki & Scalliet, 2013). A number of shifts in SDHI fungicide sensitivity have been registered under field conditions and this is partly because of the changes in disease patterns and product usage across the multiple modes of action year after year. Reduced sensitivity to SDHIs was discovered in the populations of *Alternaria alternata* on the nut crops in the USA (Avenot et al. 2008 & 2009). Reduced field efficacy of certain SDHIs was also reported in species of *A. solani* on potatoes in the US (Gudemstad et al. 2013), *Botrytis cinerea* from apple (Yin et al. 2011), kiwi (Bardas et al. 2010), strawberry (Veloukas et al. 2011), *Corynespora cassicola* on cucurbits in Japan (Miyamoto et al. 2009; Ishii et al. 2011), *Didymella bryoniae* on cucurbits in the US (Avenot et al. 2012), and *Podosphaeria xanthii* in the US (Miazzi & McGrath, 2008) and Japan (Miyamoto et al. 2009 and 2011).

Methyl benzimidazole-2-yl Carbamate (MBC), another class of fungicides is the active component of the widely used fungicide Benomyl. MBC's are very popular because of their specificity in action against target cells (Woods, 1982). The MBC has been found to cause metaphase arrest during the mitosis (Davidse, 1975; Howard et al. 1977). The

effects observed with MBC are all apparently due to the disruption of microtubules hence, the MBC fungicides affect mitosis by disrupting the microtubules (Woods, 1982).

1.13 Justification of the study

Wheat (*Triticum aestivum* L.) is one of the most important sources of food all over the world (FAO, 2017). The wheat grains are nutritive with good energy enrichment, dietary fiber, proteins, vitamins and other minerals (USDA-National Nutrient Database for Standard Reference, Release 19, 2006; FAOSTAT, 2017). In order to succeed in producing good and quality high yields of wheat, a number of management practices have to be in place right from variety selection, tillage, planting, pest management and harvesting up to storage. This is because the wheat value chain can be disrupted by a number of factors like hailstorms, pests and diseases and winter kill among others. Diseases and pests can impact wheat productivity by about 20-40% (FAO, 2012). Tan spot and stripe rust are prevalent wheat crop yield-depriving diseases world over (Sharma et al. 2003; Wellings, 2007). Tan spot disease is of great economic importance leading to increased incidence, severity and yield loss of up to 50% when ideal conditions are present (Faris et al. 2013, Hosford & Busch, 1974; Rees et al. 1981). Tan spot can be primarily managed by employing resistant cultivars and fungicide application (De Wolf et al. 1998) in an Integrated Pest Management (IPM) approach.

Stripe rust is an important disease of wheat registering up to 100% yield loss in susceptible cultivars and a 2.2 % annual yield loss (Murray et al. 1995; Chai et al. 2014, Chen, 2005). A number of studies have been carried out on the efficacy of seed treatments in the management of soil, air and seed-borne pathogens (Buck et al. 2009;

Beres et al. 2016; May et al; 2010; Shaafsma et al. 2005; Hewett & Griffith, 1986; Veron et al. 2009; Larsen et al. 2013; Hwang et al. 2015; Lipps et al. 2000). However, all these do not exhaustively address the efficacy of seed treatments in the early management of foliar diseases like tan spot and stripe rust. There remain unanswered questions as to what extent the seed treatments are translocated vertically to offer foliar protection early in the season and for how long. In addition, there are also numerous claims by chemical fungicide companies that fungicide seed treatments suppress early foliar diseases but this cannot be authenticated due to a paucity of information to back it up. Stripe rust pathogen is spread through air whereas tan spot can be air, stubble, and seed-borne but apparently, there is no substantial evidence of fungicide seed treatments conferring early season protection from foliar infections in the NGPs. Reports about the control ability of both foliar and seed-borne wheat diseases by fungicide seed treatments have been published (Mehta et al. 1992; Mehta & Fungaro, 2000). There's limited information on the longevity of protection and effect of using fungicide seed treatments singly in managing the early season foliar diseases. There are growers using the fungicide seed treatments to manage foliar diseases because of alleged double effect on both foliar and root/seed-borne diseases by different fungicide manufactures. It is also recorded that use of seed treatments is one of the least expensive disease management choices a grower can make (Mathre et al. 2001; Turkington et al. 2016; Edgington, 1980). Seed treatments are also said to have a systemic effect whereby the active ingredient is taken into the germinating seed and moves in an upward direction, protecting the seedling during development as well as after emergency (Edginton, 1980). It, however, remains unclear as to whether the fungicide seed treatments have any impact on the foliar diseases since

mostly in the U.S, the previously used surface disinfectant chemicals did not protect foliage from airborne fungal pathogens (Luz and Bergstrom, 1986). This is partly because fungicide seed treatments mainly target the soil and seed-borne pathogens (Jones, 2000; Stack & McMullen, 1988; Mehta, 1993; Sharma-Poudyal et al. 2005; Duveiller et al. 2005; Edgington, 1980). This further underscores the need to evaluate the efficacy of these compounds in managing early fungal leaf diseases of wheat.

As the global demand for organic products rises, it is clear that the wheat growers have to think through ways of holistically producing organic wheat to feed the organic pastries and other whole food markets. One of the approaches includes organic disease management. This study aimed at evaluating the efficacy of some of the certified organic biochemical and microbial pesticides in the management of tan spot in wheat under field and greenhouse conditions. This study bridges the knowledge gap regarding the efficacy of biopesticides in the management of cereal foliar diseases. The information gained would help growers make informed disease management decisions and this would ease their work in the organic wheat production value chain.

In the fight against plant disease which causes severe yield losses and reduces crop quality, there are a number of concerns especially the likelihood of developing resistance to the pesticides when used non judiciously to manage plant diseases (Van den Bosch & Gilligan, 2008.) Any fungicide resistance management strategy should aim at delaying the evolution and spread of resistance in a sensitive pathogen population (Hobbelen et al. 2014). Most fungicide sensitivity studies have targeted other pathogens and crops (Edgington et al. 1971; Avozani et al. 2014; Chen et al. 2013; Chung et al. 2009; Chapman et al. 2011). Only a few studies have been conducted to elucidate the

possible evolution of resistance/insensitivity to fungicides by *Ptr* in wheat (Patel et al. 2012; Reimann & Deising, 2005). These studies are not current enough and do not address most of the states in the Northern Great Plains (NGPS). In the NGPs, fungicides are used every growing season which calls for routine monitoring of fungicide efficacy and sensitivity in managing such wheat fungal pathogens. A couple QoI, azole, and MBC resistance cases have been reported in other fungi (Karaoglanidis et al. 2011; Kim et al. 2003; Ma & Uddin, 2009; Petkar et al. 2017; Dekker, 1976). This is a warning to the wheat pathogens like *Ptr*, hence. This study seeks to establish whether there could be some resistance in the *Ptr* pathogen from the recently collected *isolates* in the Northern Great Plains (NGP) as a result of a continued use of azoles (C_{14}-demethylase inhibitors), strobilurins (quinone "outside" inhibitors), MBCs (metaphase arrest) and the mixed mode of action fungicides. The information gathered from this study will enable growers and fungicide manufactures during pesticide disease management and chemical processing respectively.

The objectives of this study were to 1) evaluate the efficacy of fungicide seed treatments in the management of early tan spot and stripe rust of wheat. 2) evaluate the efficacy of biopesticides in the management of tan spot 3) Assess *Pyrenophora tritici repentis* isolates for sensitivity/insensitivity to commonly used fungicides in the NGP.

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CHAPTER 2

2.0 Efficacy of fungicide seed treatment in the management of early tan spot and stripe rust in wheat

Abstract

Tan spot and stripe rust caused by *Pyrenophora tritici-repentis* and *Puccinia striiformis* Westend. f. sp. *tritici* Erikss, respectively, are common foliar diseases of wheat (*Triticum aestivum L*.) with the potential to cause extensive grain yield losses. Tan spot causes 5% grain yield loss in South Dakota but individual field grain yield loss can be higher with recorded losses of up to 50%. *P. tritici-repentis* infections can start in the fall in winter wheat especially when there is extended warm weather. Similarly, extended warmer fall conditions can lead to occasionally stripe rust developing in emerging winter wheat. Some producers are using fungicide seed treatments to manage foliar fungal diseases that develop in winter and spring wheat early in the season. However, there is a paucity of information with regards to fungicide seed treatments efficacy in winter wheat to suppress foliar fungal disease in fall. To determine the efficacy of fungicide seed treatments in managing tan spot, tan spot and stripe rust susceptible spring and winter wheat cultivars 'Select' and 'Brick', 'Alice' and 'Expedition', respectively, were subjected to two fungicide seed treatments: ipconazole + metalaxyl, triticonazole + metalaxyl, and a non-treated check (naked seeds). Treated and non-treated seeds were planted into "cone-tainers" under greenhouse conditions and winter wheat cultivars planted in the fields at the South Dakota State University (SDSU) Northeast Research Farm near South shore and SDSU Volga Research Farm. Seven days and ten days after planting, all plants were inoculated with *P. tritici-repentis* and *Puccinia striiformis* f. sp.

tritici at a concentration of 3,000 conidia ml^{-1} and ~ $6x10^5$ spores/ml of spores, respectively, until runoff. The inoculated plants were placed in a misting chamber for 24 hours and then maintained in the greenhouse. Plants inoculated with *P. striiformis* f. sp. *tritici* were put in a growth chamber maintained at 17 ^oC day 12 ^oC night temperatures and 98% humidity for 48 hours. Plants were assessed for tan spot severity at 7 and 14 days after inoculation while those inoculated with stripe rust pathogen were assessed 15 days after inoculation. Field studies relied on natural inoculum sources. Results indicated a significant difference between fungicide-treated plants and the non-treated check for tan spot severity, number, and size of lesions for the greenhouse study. Tan spot severity at 2, 3, and 4 weeks after inoculation and was not significantly different among fungicide seed treatments but were significantly different from the check for disease severity, number, and size of lesions. Likewise, disease severity for stripe rust was significantly low in the treated plants than the untreated ones. Tan spot disease severity in "Redfield" cultivar was numerically lower at 14 and 20 days after planting in the treated plots at Volga. However, there was a significantly lower tan spot disease severity in "Ideal" cultivar treated plants (11-22%) than the untreated ones (48-64%). At the NERF, tan spot disease severity was significantly reduced by fungicide seed treatments in both cultivars at 14 and 20 days after planting. Plots with fungicide seed treatments had a high plant density, winter survival rate and a higher grain yield as compared to plots with no seed treatments at both locations. These results indicate fungicide seed treatments may be useful in reducing early tan spot and stripe rust diseases in wheat.

2.1 Introduction

Wheat is one of the most widely cultivated staple grains globally and is a key source of calories and proteins needed by the human body (Curtis et al. 2002; Breiman & Graur, 1995). The production of wheat is faced with a number of biotic and abiotic constraints. Plant diseases are one of the biotic problems faced by wheat (Heydari & Pessarakli, 2010). Use of fungicide seed treatments has proved to be an important strategy not only for ensuring optimal stand but also lessening early-season foliar fungal diseases (Menzies & Gilbert, 2003; Wiese, 1987). Some of the diseases controlled by fungicides include tan spot caused by *Pyrenophora tritici-repentis* and *Puccinia striiformis* Westend. f. sp. *tritici* Erikss.

Pyrenophora tritici-repentis is a stubble-borne disease with yield losses ranging between 5- 50% (Singh et al. 2010; Shabeer and Bockus, 1988). In South Dakota, 5% grain yield loss has been recorded but individual field grain yield loss can be higher up to 30 % (Buchneau et al. 1983). In efforts to curb down tan spot effects on wheat yield, a number of measures such as breeding for resistance, use of fungicides, and cultural practices including crop rotation and stubble management are being used. Likewise, stripe rust is an important disease of wheat world over (Hovmøller et al. 2011; Wellings, 2011) and in South Dakota (Byamukama, 2015). The effect of stripe rust on the susceptible wheat cultivars can be severe especially if infection occurs before booting (Boshoff, 2003). Stripe rust has been reported to cause grain yield losses of between 50-100 %, a reduction in kernel mass of 43% and a decrease in the kernel number of 72% (Murray et al. 1995; Chen, 2005).

Fungicide seed treatment is used by growers to manage seed-borne and soil-borne pathogens that attack seed and seedlings. However, it has been recorded that fungicide seed treatments are used by some growers with anticipation to manage the foliar diseases such as tan spot, spot blotch, and stripe rust among others that show up early in the wheat growing season (Stack and McMullen, 1988, Mehta, 1993, Sharma-Poudyal et al. 2005, Duveiller et al. 2005; Boshoff, 2003). Most fungicide seed treatments are applied as pre-coated on the grains and are taken up by the seedling at germination and later distributed throughout the leaves and other plant tissue depending on the mode of action (Sierotzki & Scalliet, 2013; Davidse, 1975; Howard et al. 1977; Buchenauer,1987; Lyr, 1995). Fungicide seed treatments act as pathogen repellents or antagonists hence reduce pathogen infection, reduce sporulation and increase the pathogens' latent period. Seed treatments inhibit surface and internally seed-borne pathogens. Soil-borne pathogens like Pythium, Rhizoctonia, and Fusariums are highly inhibited by fungicide seed treatments (Wegulo, 2017). Studies by Sharma-Pourdyal et al (2005 & 2016) indicated that Helminthosporium leaf blight disease complex was effectively managed in spring wheat by seed treatments when a combination of triadimenol + carboxin + thiram was used. Sharma-Poudyal et al (2005) also reported increased kernel weight and grain yield due to the use of propiconazole fungicide seed treatment. Other studies have indicated that Helminthosporium leaf blight disease complex was effectively managed in spring wheat by seed treatments using a combination of triadimenol and carboxin + thiram with a 9% and 8 % grain yield increase, respectively (Sharma-Purdyal et al. 2005 & 2016). In the case with tan spot, seed treatments with a foliar-active systemic action will confer additional disease control and allow a good complementation of the subsequent foliar

applications for instance in case of a delay of foliar treatment (Bartlett et al. 2002). Luz, (1986) indicated that tan spot was reduced by traideminol seed treatment 20-30 days after sowing under controlled environmental conditions and this is one of the motivations for most growers to use seed treatments. In stripe rust management, seed treatment products containing triademenol or triticonazole gave protection to plants against stripe rust for about 4 weeks after sowing (Hollaway, 2018). Other studies indicating successful seed treatment for the control of wheat diseases like smuts, kernel bunt, and root rots are; imazalil, nuarimol, triadimenol, propiconazole, difenoconazole, and flutriafol (Stack and McMullen, 1991), epoxiconazole (Sharma et al. 2005); tebuconazole, ciproconazole, fluzilazole, metaconazole and propiconazole (Viedma & Kohli, 1998); triademefon, fentinacetate-maneb and propiconazole (Hobbs et al. 1998; Lapis, 1985). Larsen and Falk, (2013) reported that a dual fungicide (difenoconazole & metalaxyl) and an insecticide (thiamethoxam) enhanced the frost tolerance of wheat seedlings hence increased winter wheat survival which ultimately boosted yield.

Although several studies have reported benefits to fungicide seed treatments with regards to early foliar fungal diseases control, no information is available on the effectiveness of this practice in the Northern Great Plains region. One additional benefit due to seed treatments in winter wheat is the increased plant vigor that leads to better winter survival (Turkington et al. 2016). Fungicide seed treatments have also been reported effective in improving the winter wheat stand establishment (Schaafsma & Tambauric llincic, 2005; Menzies & Gilbert, 2003; Wiese, 1987). There is limited to no information on the effect of fungicide seed treatment in managing early foliar diseases in winter and spring wheat, especially in the frigid Northern Great Plains environment. Also, a number of previous

studies did not fully address the duration of protection by fungicide seed treatments and the possible impact these have on subsequent diseases and ultimately the yield. Thus, the objectives of this study were to 1) Determine the efficacy of fungicide seed treatments in the management of early tan spot and stripe rust diseases in wheat 2) Assess effect of fungicide seed treatment on winter stand establishment and winter survival 3) Determine the effect of fungicide seed treatments on yield.

2.2 Materials and methods

Studies to establish the efficacy of fungicide seed treatments in the management of tan spot and stripe rust were conducted both in the greenhouse and in the field. Two winter wheat cultivars were evaluated using different fungicide treatments at different planting times i.e., early and late fall planting for the field studies.

2.2.1 Greenhouse study

Greenhouse experiments were conducted at the South Dakota State University Plant Science greenhouse. Hard red spring wheat cultivars "Select" and "Ideal", and hard red winter wheat cultivars; "Alice" and "Expedition" that have varying susceptibility to tan spot and stripe rust were planted in the greenhouse experiments. The spring and winter wheat seeds were treated with two fungicides with active ingredient combinations difenoconazole + mefenoxam + fludioxonil + sedaxane (Warden Cereals WR11, WinField® United) and pyraclostrobin + triticonazole + metalaxyl (Stamina F^3, BASF, U.S) and the untreated naked seeds were the untreated check. The study was arranged in a complete randomized design and treated and untreated seeds planted in separate "cone-tainers" (Stuewe & Sons, Inc. Tangent, OR) of a cell diameter of 3.8cm and a depth of

20cm filled with a soil mix pro-mix® BX mycorrhizae (Greenhouse Megastore, Danville, IL). Five seeds per cone for each of the cultivars were planted and thinned to four seedlings after emergence.

2.2.1.1 Inoculation and disease ratings

The *Pyrenophora tritici-repentis* (*Ptr*) race 1 "*Pti2"* isolate was used for the greenhouse inoculations. The isolate was obtained from wheat samples collected in South Dakota and kept in the freezer at -20^{o}C. A fresh culture of the isolate was initiated on by growing plugs on V8-PDA (V8 juice: 150 ml; $CaCO_3$: 3 grams, Potato Dextrose Agar: 10 grams; Agar 10 grams; distilled water 850 ml) (Lamari & Bernier, 1989). After 5 days of incubation under dark conditions, the V8-PDA plates with growing mycelia were matted down using a flame-sterilized test tube bottom and transferred to light for 24 hours followed by another round of 24 hours' darkness at 16 ^{o}C to facilitate conidia formation. Using a sterilized wire loop, conidia were scrapped off the media and counted on a microscope. Conidia spores were brought to a concentration of 3000 spores/ml before inoculation. The diluted spores were transferred to a Preval sprayer (Nakoma Products, Bridgeview, IL) and all plants were inoculated until runoff. To determine the length of fungicide seed treatment protection against tan spot development, tan spot pathogen inoculations were done at 7, 14, 21 and 28 days after planting. The inoculated plants were transferred to the humidity chamber set to mist for 10 seconds every after 12 minutes for a span of 24 hours to enhance infection (Lamari & Bernier, 1998). After 24 hours in the misting chamber, the rack containing inoculated plants was transferred to the greenhouse bench. Rating for the percent disease severity, lesion size and number was conducted 7 and 14 days after inoculation (DAI).

For stripe rust inoculation, wheat seedlings were inoculated with *P. striiformis* f. sp. *tritici (Pst)* urediniospores collected in 2017 from Brookings county Crop Performance Trail (CPT). The spores were kept in a freezer at -80 ^{o}C. Frozen spores were recovered and heat shocked in a water bath at 45 ^{o}C for 2 minutes. At 10 days after planting, the seedlings were spray inoculated with the recovered urediniospores at a concentration of ~ $6x10^5$ spores/ml of spores suspended in soltrol 170 oil (Phillips Petroleum, Bartlesville, OK) at a rate of 0.01g/mL and then left to air dry for 30 minutes. The seedlings were later transferred to the dew chamber set at 10 ^{o}C and supplied with 98 % humidity for 48 hours in the dark. The plants were maintained in a growth chamber at 17 °C/ 12 °C (day/night) at a 16-hour photoperiod in a growth chamber. Disease severity was assessed 20 days' post inoculation based on the total percentage leaf area diseased (TPLAD). The experiments ran for 4 weeks for the spring wheat tan spot study and 6 weeks for the winter wheat stripe rust study. Tan spot inoculations were done every week so as to establish the length for which fungicide seed treatments are active against tan spot pathogen. Both stripe rust and tan spot greenhouse studies were repeated twice.

2.2.2 Field study

Two winter wheat cultivars "Ideal" and "Redfield" were planted at South Dakota State University (SDSU) Northeast Research Farm (NERF) near South Shore and at SDSU Volga Research Farm in fall 2017. These cultivars were selected for varying in susceptibility to tan spot and stripe rust with Redfield being moderately susceptible and Ideal is susceptible (Kleinjan et al. 2015).

2.2.3 Fungicide treatments and study design

Winter wheat was sown into the previous season's wheat stubble to facilitate tan spot infection. The following fungicide seed treatments and rates in milliliters per hundred-pound weight (cwt) were applied to the cultivars "Redfield" and "Ideal". prothioconazole + penflufen + metalaxyl (Evergol® Energy, Bayer® CropScience, Research Triangle Park, NC) at 29.6 ml; sedaxane (Vibrance® 500FS, Syngenta® USA) at 2.4 ml; pyraclostrobin (Stamina®, BASF, Research Triangle Park, NC) at 26ml; ipconazole+ metalaxyl (Rancona® Pinnacle, MacDermid Agricultural Solutions, Inc. Waterbury, CT) at 146 ml; difenoconazole + mefenoxam (Dividend® Extreme, Syngenta® USA) at 118 ml and the untreated check (naked seed). The study design was a split-plot design with cultivar as the main plot factor and fungicide treatments as the subplot and treatments were replicated four times. To assess time of planting effect on the effectiveness of fungicide seed treatments, two planting times were done: September 9th for early planting and October 24th for late planting at the Volga location and on September 8th and October 25th for the NERF for the early and late planting, respectively. The plots were planted with a 7-row tractor mounted small grain planter fitted with cone units at a seeding rate of 323 seeds/m^2. Plot size was 1.5m wide by 4.6m long. Stand counts were done 8 and 14 days after planting for the early planting and 10 days after emergence in late spring for the late planting. Foliar disease rating was conducted 10 days at the two emerged leaves in late fall and at 10 and 20 days after emergence and in late spring. Disease severity ratings were recorded for the first two lower leaves and flag leaves for the fall ratings. Percent disease severity was recorded for each leaf based on chlorotic and necrotic lesions for the 20 randomly selected plants per plot. Further, plant density counts

and height measurements (cm) were conducted in the late spring of 2018 on the late-planted plots at Northeast Research Farm (NERF) and Volga to ascertain winter survival at 20 days after planting. The plant stand counts were done using 1000mm hula-hoop for both the early and late planted plots at the Volga research farm. A measuring tape was used for the late planted plots (at 1m length) at the NERF due to poor emergence as a result of winter kill. Foliar disease rating was conducted 14 and 20 days after planting for the early planted plots. Total leaf disease severity was rated for each leaf as the total percentage leaf area covered with tan spot lesions for 20 randomly selected plants per plot. At all the locations, plots were harvested using a small plot combine and yield per plot, test weight and protein content were recorded.

To assess the incidence of root rot pathogens for different treatments, ten plants were randomly uprooted per plot at seedling and early booting stages and were cleaned by washing soil off the plant roots using running tap water. The plants were wrapped in paper towels and left to dry overnight to remove any excess moisture. Plant crown roots were excised using a sterile pair of scissors from each of the ten plants to a length of 0.5-0.7cm. The root segments were surface sterilized in 1% bleach for 60 seconds and rinsed in double distilled water for 60 seconds. The sterilized root segments were plated on a lactic acid-amended half strength PDA. Each plate had 4 spaced root segments which were left to incubate at room temperature ranging from 20-23 ^{o}C with 12-hour alternate dark and light conditions for 7 days. The 7 day-old cultures from each of the 4 root segments per plate were individually transferred using a flame-sterilized scalpel onto fresh half strength PDA plates to get pure cultures of the fungi. The fungal growth was assessed using a compound Zeiss microscope illuminated with an illuminator 100 (Carl

Zeiss Inc., Thornwood, NY) for identification. The percent pathogen incidence was determined for each root rot as follows: The number of root pieces with the particular pathogen/total number of root pieces per plot x 100.

2.3 Data analysis

Disease severity, number of lesions, size of lesions, and height of plants data were subjected to multiple linear model procedures in the R computing environment (R Core Team, 2018) to determine the effect of seed treatments in controlling tan spot and stripe rust and also on yield and plant vigor. Two greenhouse repeats were combined after conducting homogeneity of variance test.

For statistical models, fungicide seed treatments and cultivar for both the greenhouse and field studies were considered fixed factors whereas location, plots and "cone-tainers" were the random factors. Analysis of variance (ANOVA) was conducted for both greenhouse and field studies on the percentage disease severity, an average number of lesions, size of lesions and only percentage disease severity for the stripe rust study. From the analysis of the main and interactive effects, means were separated using Fisher's Least Significant Difference. The data for root disease assessments were log transformed to minimize variance and meet the normal distribution assumptions.

2.4 Results

2.4.1 Efficacy of seed treatment to manage tan spot in the greenhouse study

The ANOVA revealed significance fungicide seed treatments effect ($P \leq 0.05$) for, weeks after planting, cultivar and the rating interval (days after inoculation) effects

on disease severity, lesion numbers, and size (Table 2.1). Since weeks after planting-treatment effects were significantly different, means are analyzed by weeks after planting (Table 2.2). Significant differences occurred between treatments for the four assessment dates. Disease severity was high in plants inoculated at 2, 3 and 4 weeks after planting and was low in 1 week after planting. Likewise, the number of lesions significantly varied across treatments 2, 3 and 4 weeks after plating. There was a reduction in the efficacy of the fungicide seed treatments as wheat seedlings' growth progressed from week 1 to week 4 in terms of disease severity. Overall, there was a significantly low disease severity, size and number of lesions for the fungicide seed-treated plants as compared to the untreated (Table 2.2). Products pyraclostrobin + triticonazole + metalaxyl and thiamethoxam+ difenoconazole + mefenoxam + fludioxonil + sedaxane were not significantly different from each other. There were numerical differences between fungicide treatments in controlling tan spot severity with thiamethoxam + Difenoconazole + mefenoxam + fludioxonil + sedaxane treated plants having lower disease severity scores compared to pyraclostrobin + triticonazole + metalaxyl.

2.4.2 Efficacy of seed treatment to manage stripe rust in wheat in the growth chamber

Two runs were combined for the stripe rust study. Disease severity was significantly different amongst treatments with the untreated (naked) seeds having a high percentage of disease severity of 53% (Table 2.4) compared to 41 and 36% for seed treated plants. Pyraclostrobin + triticonazole + metalaxyl (Stamina® F^3) and difenoconazole + mefenoxam (Dividend® Extreme) were not significantly different from each other in controlling stripe rust disease. However, plants treated with pyraclostrobin + triticonazole + metalaxyl (Stamina® F^3) had numerically lower disease severity than

difenoconazole + mefenoxam (Dividend® Extreme). There was no significant difference between treatments and cultivar (Table 2. 3). Also, there was no treatment-cultivar interaction hence the disease severity results were independent of the wheat cultivars used in the study.

2.4.3 Efficacy of seed treatments in managing tan spot under field conditions

There was no significant difference in the planting time and treatment interaction for disease severity hence both the early and late planted data for the plots was combined for analysis. Disease severity was significantly different amongst cultivars, seed treatments, and locations. At Volga research station, treatments did not significantly differ in disease severity both at 14 and 20 days after planting. "Ideal" cultivar had a significantly high tan spot infection (percent severity) in the untreated plants with 48.5% and 63.6% at 14 and 20 days after planting, respectively (Table 2.5). Tan spot severity was not significantly different amongst the fungicide seed treatments but there were numerical differences with difeconazole + mefenoxam treated plants having the least disease severity at 14 days after emergence (DAE), and ipconazole + metalaxyl, sedaxane and pyraclostrobin at 20 DAE for the "Ideal" cultivar. At the Northeast Research Farm (NERF) disease severity was high in the untreated plots at 13.5 and 34.1% at 14 and 20 DAE in "Redfield" cultivar and 28.4 and 38.6% at 14 and 20 DAE, respectively, for the "Ideal" cultivar. The tan spot severity for plots under seed treatments was not significantly different for "Redfield" cultivar at NERF at $\alpha = 0.05$ level of significance but was numerically different with sedaxane and difeconazole + mefenoxam that had the least percent disease severity. In the "Ideal" cultivar the seed treatments were significantly different in controlling tan spot with the least disease severity from

pyraclostrobin (7.7%, 13.3% at 14 and 20 DAE respectively), Sedaxane (11.2% at 14 DAE) and prothioconazole + penflufen + metalaxyl treated plants (4.9%, 12.8% at 14 and 20 DAE respectively) (Table 2.5). For all the plant densities, the untreated plots had low plant counts compared to the treated plants (Table 2.5). The mean plant densities were high relative to non-treated check for the plants treated with difeconazole + mefenoxam at 8 DAE and prothioconazole + penflufen + metalaxyl at 16 DAE. Winter survival had significant location and treatment interaction (Table 8) and the results for both locations indicated untreated plants with low plant densities (Table 2.9) compared to the treated plants. There were no significant differences for plant height between the treated and untreated plants but there were numerical differences where the untreated plots had shorter height measurements (cm) as compared to the seed treated plants (Table 2.6). Overall, plants at the Northeast Research Farm had high plant densities. The yield was not significantly different amongst fungicide seed treatments for the early planted plots but was significantly lower in the check, for both the early and late plantings. The late planting had lower yields but there were significant differences amongst the treatments with prothioconazole + penflufen + metalaxyl (Evergol® Energy) having higher yields (Table 2.7). There was a positive correlation between yield and the disease rated at 20 DAE for the early and late planted plots.

For root diseases analyses at different plant stages, the plants at the seedling stage were not significantly different for *Bipolaris Sorikiniana* and Rhizoctonia root rot. The recovery rate of Fusarium species was significantly high in the untreated plots at the seedling stage (Table 2.10) but there were no significant differences amongst the rest of the treatments including in the early booting stages. The plants at early booting stages

similarly had a higher *Bipolaris Sorikiniana* recovery rate (incidence) in the untreated plots followed by plants treated with sedaxane, prothioconazole + penflufen + metalaxyl, and the least incidence in pyraclostrobin, ipconazole + metalaxyl and difeconazole + mefenoxam. Likewise, there was no significant difference in the incidence of Fusarium and Rhizoctonia root rot disease at the early booting stage but Fusarium had comparably high incidence than Rhizoctonia. Overall, there was more Fusarium *spp* disease incidence at the early booting stage of growth than the seedling stage. At all stages, Rhizoctonia was the least recovered.

2.5 Discussion

The results from the greenhouse and growth chamber for assessment of the effect of seed treatments in the management of early tan spot and stripe rust diseases respectively indicated a significant reduction in the percent disease severities. This was from a comparison between treated and untreated plants inoculated at different weeks after planting for tan spot and inoculations at two weeks old plants for stripe rust in the greenhouse studies. Winter wheat field plots also indicated a significant reduction of tan spot in the seed treated plots from NERF and Volga research farms early in the season. Fungicide action on the tan spot pathogen spores might have been through the systemic movement of the fungicide into young wheat leaves since there are records of the vertical movement of the active ingredients to offer protection to the aerial parts through root absorption (Rowell, 1976). This also matches studies by Sharma-Pourdyal et al (2005 & 2006) where Helminthosporium leaf blight complex which includes tan spot was effectively managed using seed treated with triadimenol + carboxin + thiram. There was a general increase in disease severity for the 2, 3 and 4 weeks old plants in the

greenhouse and 20 days old plants for the winter wheat field plants. The disease severity was significantly high in the control as compared to the treated plots and this might be because the fungicides do reduce infection by eliminating or decreasing the amount of secondary inoculum under field conditions.

The mean plant density/m^2 and height was high in the treated plots especially in those treated with difeconazole + mefenoxam under field conditions. This might be because the seed treatments enhanced seedling emergence and also offered protection from the root diseases. The results of increased plant density are consistent with studies conducted by Turkington et al (2016) and Beres et al (2016) where plant density increased with seed treatment application. Other related studies by (Reis, 1991; Herrman et al., 1990; Stack and McMullen, 1991; Giri et al. 2001) also reported increased germination when seed treatments were applied.

Poor plant stand at the NERF location was most likely due to poor snow cover and late planting reasons hence the fungicides had not much of an effect in improving plant stand and vigor. A study by Turkington et al (2016) reported poor stands because of poor snow cover in a study from one of the provinces in Manitoba, Canada. The winter survival in terms of plant density was significantly different for both studies at Volga and NERF with a high density in the treated plots.

The higher yields in the treated plots than untreated ones are attributed to good plant vigor and disease protection early in the season by the fungicides. A study, in Ontario, Schaafsma, and Tamburic- Ilincic, (2005) reported that most fungicide seed treatments increased yield. This study also verified the results of Sharma-Pourdyal (2005) where propiconazole treated plots increased kernel weight and grain yield. Another study

though in soybeans by Bradley, (2008) reported that fungicide seed treatments with active ingredients fludioxonil + mefenoxam and azoxystrobin + metalaxyl prevented stand and yield losses, especially under cool and moist soil condition just like was the case for the plots at NERF and Volga Research Farms that were treated with fungicide seed treatments. On a different note, the generally low (from the expected according to the seeding rate of 323 seeds/m^2) but significantly different yields from the late-planted plots were due to poor winter survival, lodging, and poor seed emergence at both locations. A study in South Africa by Boshoff et al (2003) where fungicides triadimenol and triticonazole were applied as seed treatments resulted in high yields and reduced stripe rust diseases in wheat. This partly explains the high yields in field plots with fungicide seed treatments and the low disease severity ratings for the stripe rust disease conducted in the growth chamber where treatments pyraclostrobin + triticonazole + metalaxyl (Stamina® F^3) and difenoconazole + mefenoxam (Dividend® Extreme) was applied. Also, the study agrees with Hollaway (2018) who noted that seed treatments containing triademenol or triticonazole offered protection to plants against stripe rust for close to 4 weeks after sowing. A similar study by Luz and Bergstrom (1986) indicated both disease control and yield increase in plants treated with triadimenol while managing tan spot, powdery mildew, spot blotch and septoria nodurum spot in spring wheat. A study by Mendham et al (2009) showed that winter-oil seed rape *(Brassica napus)* planted early in autumn yielded more than the late spring planting.

For the root diseases, only *Bipolaris Sorikiniana, Fusarium spp,* and *Rhizoctonia* spp were isolated from diseased roots in the late planted trials from Volga and NERF. In this study, there was a considerably lower recovery rate of *Bipolaris sorikiniana* and

Rhizoctonia in plots treated with ipconazole + metalaxyl at the early booting stage of wheat. Strobilurins like pyraclostrobin and azoxystrobin have been reported to have control effects on *Fusarium*, *Rhizoctonia*, *Phytophthora*, and *Pythium* spp. (Barlett et al. 2002; Broders et al. 2007; Hewitt, 1998; Kiewnick et al. 2001). Fludioxonil was also reported to have activity on *Rhizoctonia* and *Fusarium* spp. (Hewitt,1998; Broders et al. 2007; Meyer et al. 2006; Munkvold et al. 2002) and this might explain the low recovery rates in these two pathogens from our study in the fungicide-treated plots. Other reports of several fungicides such as iprodione + thiram or iminoctadine (Reis, 1991); imazalil (Herrman et al. 1990); imazalil, nuarimol, triadimenol, propiconazole, difenoconazole, and flutriafol (Stack & McMullen, 1991); mancozeb and thiram (Giri et al. 2001); carboxin + thiram, and carbendazim (Sharma-Poudyal et al. 2005) have been reported to be useful in protecting germinating seeds and seedlings from early infection.

The seed treatment plots displayed significant grain yield differences as compared to the untreated ones. In a recent study by Turkington et al (2016), grain yield was improved in spring wheat by application of foliar fungicides but there was no recorded synergistic or antagonistic effect of improved yield with the application of seed treatments. Also, in order for a fungicide to effectively impact yield, a fungicide application should protect the flag leaf (Fernandez et al. 2014, Turkington et al. 2004 &2015, Poole & Arnaudin, 2014). However, our results might explain the systemic protection accorded by the seed treatments over time. This might be one reason for the observed yield in the treated plots than the untreated. Previous studies indicate that seed treatments will not benefit yield where no seed/soil-borne diseases do not exist (Heer, 1998, Guy et al. 1989, May et al. 2010) but this study went further to isolate soil-borne

pathogens in the late planted plots and there was a great pathogen incidence evidenced by the recovery/isolation rate in the untreated plots as compared to the treated plots (Table 2. 10). This gives confidence that seed treatment had an impact on the soil-borne diseases and ultimately impacted yield, for example, a higher yield was registered in pyraclostrobin treated plots, and likewise, there had been a higher incidence of the common root rot (Tables 2.7 and 2.10).

The findings of this study indicated that use of seed treatments would manage an early foliar disease like tan spot and stripe rust in wheat. The study also elucidated that there were higher wheat winter recovery and plant density in plots with fungicide-treated seeds. Ultimately the yield benefit was recorded in seed treated plots. An integrated management approach to early foliar diseases has benefits of managing soil and seed-borne diseases as early wheat foliar diseases.

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Table 2.1: Analysis of variance (ANOVA) for the number of lesions, size of lesions and percent disease severity for two fungicide seed treatments and the untreated plants as observed for four weeks in the greenhouse studies in 2017 and 2018

Dependent variable, Source of variation	**DF[a]**	**MS**	**P>F**
Disease severity			
Seed Treatment	2	34524	< 0.001
Cultivar	1	7035	< 0.001
Weeks after planting	3	44896	<0.001
WAP*Treatment[b]	6	2985	<0.001
Number of lesions			
Seed Treatment	2	7785.4	<0.001
Cultivar	1	20087.1	<0.001
Weeks after planting	3	19593.2	<0.001
WAP*Treatment	6	603	<0.001
Size of lesions			
Seed Treatment	2	3.817	<0.001
Cultivar	1	0.023	<0.001
Weeks after planting	3	57.808	<0.001
WAP*Treatment	6	0.266	0.5012

[a]Degrees of freedom
[b]WAP*Treatment Weeks after planting and treatment interaction
Data was combined across the two runs after the test of homogeneity of variance

Table 2.2: Effect of fungicide seed treatments on tan spot severity, number of lesions, size of lesions at different days after planting for the pooled 2017 and 2018 greenhouse study

Weeks After Planting, Treatment	**Disease severity (%)**		**Number of lesions**		**Size of lesions (cm)**	
week 1						
Check	54.5	a	32.1	a	0.60	a
Pyraclostrobin	39.4	b	23.4	b	0.50	ab
Thiamethoxam + difenoconazole + mefenoxam + fludioxonil + sedaxane	38.4	b	22.1	b	0.45	b
week 2						
Check	70.0	a	33.1	a	0.60	a
Pyraclostrobin	46.6	b	23.4	b	0.50	ab
Thiamethoxam + difenoconazole + mefenoxam + fludioxonil + sedaxane	43.2	b	22.0	b	0.50	b
week 3						
Check	72.3	a	41.3	a	0.53	a
Pyraclostrobin	61.3	b	34.1	b	0.48	a
Thiamethoxam + difenoconazole + mefenoxam + fludioxonil + sedaxane	57.1	b	31	b	0.44	a
week 4						
Check	69.3	a	39.3	a	0.70	a
Pyraclostrobin	65.3	ab	35.41	b	0.55	ab
Thiamethoxam + difenoconazole + mefenoxam + fludioxonil + sedaxane	62.7	b	34.1	b	0.53	b

Values are least squared means of 32 replications for the two runs and two varieties. Runs and cultivars combined after homogeneity of variance test and interaction F-values, respectively. For each treatment within a column, means followed by a common letter are not significantly different according to least-square means *t*-tests ($P \leq 0.05$)

Table 2.3: Analysis of variance table for stripe rust disease severity under different seed treatments

Variable	**[a]DF**	**MS**	**P>F**
Treatment	2	2894	<0.001
Cultivar	1	29.3	0.6673
Treatment*Cultivar	2	73.73	0.6279

[a] Degrees of freedom

Table 2.4: Mean percent stripe rust disease severity for the pooled data for run 1 and 2 for stripe rust seed treatment efficacy study

Treatment	**Disease severity(%)**
Check	52.900 a
Difenoconazole + mefonoxam	41.375 b
Pyraclostrobin + triticiconazole+ metalaxyl	36.300 b

Values are the least squared means of 40 replications for the two runs and cultivars.
For each treatment within a column, means followed by a common letter are not significantly different according to Fishers Least Significance Difference test ($P \leq 0.05$)

Table 2.5: Effect of different fungicide seed treatments on the percentage tan spot severity (percentage) for Volga and Northeast Research Farms (NERF) sorted by cultivar

Location, Cultivar, and Treatment	**Disease severity (%)**			
	14 DAE[a]		**20 DAE**	
Volga				
Redfield				
check	15	a	28	a
Prothioconazole + penflufen + metalaxyl	14	a	20	a
Ipconazole+ metalaxyl	13	a	24	a
Sedaxane	12	a	16	a
Pyraclostrobin	12	a	21	a
Difenoconazole + mefenoxam	10	a	14	a
Ideal				
check	48	a	64	a
Prothioconazole + penflufen + metalaxyl	17	b	24	b
Ipconazole+ metalaxyl	16	b	22	b
Sedaxane	13	b	22	b
Pyraclostrobin	12	b	22	b
Difenoconazole + mefenoxam	11	b	24	b
NERF				
Redfield				
check	13	a	34	a
Prothioconazole + penflufen + metalaxyl	5	b	11	b
Ipconazole+ metalaxyl	6	b	9	b
Sedaxane	5	b	9	b
Pyraclostrobin	6	b	9	b
Difenoconazole + mefenoxam	6	b	8	b
Ideal				
check	28	a	39	a
Prothioconazole + penflufen + metalaxyl	5	c	13	c
Ipconazole+ metalaxyl	13	b	22	bc
Sedaxane	11	bc	25	b
Pyraclostrobin	8	bc	13	c
Difenoconazole + mefenoxam	13	b	25	b

Values are least squared means of 32 replications over two different planting times and locations. Different letters in the same column for each treatment represent significant differences according to Fishers Least Significant Difference test ($P \leq 0.05$).

[a]DAE Days after Emergence

Table 2.6: Mean winter plant density and height for combined locations Volga and Northeast Research Farms, early and late planted plots

Treatment	Plant Density /m2 16 DAE	Plant Density/m 28 DAE[a]	Height (cm)
Check	83 a	52 a	7 a
Difenoconazole + mefenoxam	90 a	71 a	8 a
Prothioconazole + penflufen + metalaxyl	91 a	59 a	8 a
Ipconazole+ metalaxyl	88 a	58 a	8 a
Pyraclostrobin	86 a	59 a	9 a
Sedaxane	85 a	60 a	8 a

Values are least squared means of 32 replications over two different planting times and locations. For each treatment within a column, means followed by a common letter are not significantly different according to Fishers Least Significance Difference Test ($P \leq 0.05$)
[a]DAE Days after Emergence

Table 2.7: Effect of different fungicide seed treatments on yield for field plots planted early (9/8th /2017) and late (10/24th /2017) for Northeast and Volga Research Farm

Time, Treatment	Yield kg/ha	
Late planting		
Pyraclostrobin	1033	a
Prothioconazole + penflufen + metalaxyl	844	ab
Sedaxane	790	ab
Ipconazole+ metalaxyl	771	ab
Difenoconazole + mefenoxam	727	a
check	563	b
Early Planting		
Pyraclostrobin	1777	a
Prothioconazole + penflufen + metalaxyl	1676	a
Sedaxane	1768	a
Ipconazole+ metalaxyl	1804	a
Difenoconazole + mefenoxam	1724	a
check	1327	b

Values are the least squared means of 32 replications for the pooled data from all planting times and locations.
For each treatment within a column, means followed by a common letter are not significantly different according to least-square means *t*-tests ($P \leq 0.05$)

Table 2.8: Analysis of variance table for winter survival in the late planted plots at Volga and Northeast Research Farm to determine the effect of seed treatment on winter survival

Variable	**Df[a]**	**MS**	**P>F**
Location	1	1060	<0.01
Treatment	5	3228.3	<0.001
location*Treatment[b]	5	731	<0.001

[a]Degrees of freedom
[b]location*Treatment location treatment interaction effect

Table 2.9: Mean plant density/m^2 and height (cm) taken early May 2018 to estimate winter survival of late planted plants under different fungicide seed treatments sorted by location

Location, Treatment	**Plant density/m^2**	**Height (cm)**
NERF[a]		
Check	45 c	4.1 a
Difenoconazole + mefenoxam	69 b	4.1 a
Ipconazole+ metalaxyl	88 a	4.8 a
Prothioconazole + penflufen + metalaxyl	87 a	4.2 a
Pyraclostrobin	97 a	4.5 a
Sedaxane	95 a	4.3 a
Volga		
Check	68 b	9.1 a
Difenoconazole + mefenoxam	92 a	9.5 a
Ipconazole+ metalaxyl	87 a	9.4 a
Prothioconazole + penflufen + metalaxyl	94 a	9.5 a
Pyraclostrobin	90 a	9.6 a
Sedaxane	90 a	9.3 a

Values are least squared means of 32 replications over two different locations. Different letters in the same column for each cultivar represent significant differences according to Fishers Least Significant Difference test, ($P \leq 0.05$)
[a]NERF Northeast Research Farm

Table 2.10: Percentage recovery rate of root rot pathogens amongst different treatments for the late planting at different growth stages pooled for Northeast and Volga research farms

Stage, Treatment	**Percent *Bipolaris sorikiniana* (log_{10}+10)**	**Percent Fusarium spp (log_{10}+10)**	**Percent Rhizoctonia (log_{10}+10)**
Seedling stage			
Check	2.0 a	1.7 a	1.1 a
Sedaxane	1.6 a	1.2 b	1.0 a
Prothioconazole + penflufen +metalaxyl	1.6 a	1.3 b	1.0 a
Pyraclostrobin	1.6 a	1.1 b	1.0 a
Ipconazole + metalaxyl	1.6 a	1.3 b	1.0 a
difenoconazole + mefenoxam	1.5 a	1.3 b	1.0 a
Early Booting			
Check	2.0 a	2.0 a	1.1 a
Sedaxane	1.5 ab	1.7 a	1.0 a
Prothioconazole + penflufen + metalaxyl	1.5 ab	1.8 a	1.0 a
Pyraclostrobin	1.5 ab	1.7 a	1.0 a
Ipconazole + metalaxyl	1.4 b	1.9 a	1.0 a
Difenoconazole + mefenoxam	1.4 b	1.8 a	1.0 a

Values are least squared means of 16 replications over two locations for the late planted plots. Different letters in the same column for each treatment represent significant differences according to Fishers Least Significant Difference test ($P \leq 0.05$). Treatments with the same letters are not significantly different from each other.

CHAPTER 3

3.0 Efficacy of biochemical and microbial pesticides in the management of tan spot in wheat

Abstract

Use of USDA-approved chemical control of fungal pathogens in an organic wheat production system is on the rise due to an increasing demand for organic wheat products. However, information on the efficacy of such chemicals in wheat is limited. To determine the efficacy of biochemical and microbial pesticides in the management of tan spot in wheat, in-vitro, greenhouse and field studies were done. In vitro studies evaluated the growth of *Pti2* isolate of *Pyrenophora tritici repentis* on biochemical and microbial pesticides amended Petri plates to assess possible inhibition of mycelial growth. The greenhouse study used a tan spot susceptible "Select" cultivar and plants were treated with the biochemical and microbial pesticides and then inoculated with tan spot pathogen at 3 and 6 weeks after planting for the greenhouse. The field study had two hard red spring wheat cultivars "Select" and "Ideal" with varying tan spot susceptibility and were grown under natural *Pyrenophora tritici repentis* inoculum. The greenhouse plants and field plants were pre-treated with microbial and biochemical pesticides namely *Bacillus amyloquafasciens* D747, *Streptomyces lydicus* Wyec 108, *Bacillus subtilis* QST713 and hydrogen peroxide + peroxyacetic acid at two different intervals prior to inoculation. A positive check consisted of plants treated with pyraclostrobin, a proven very good synthetic fungicide for tan spot management. Both greenhouse and field plants were assessed for tan spot severity and field plots yield was recorded. Results from the greenhouse studies showed significantly low levels of tan spot severity in the treated

plants over the untreated plants and smaller (diameter) to no mycelial growth on the biopesticides-amended plates especially on treatments *Bacillus subtilis* QST713, and *Bacillus amyloquafasciens* D747 followed by *Streptomyces lydicus* Wyec 108 in vitro. There was no significant effect of hydrogen peroxide + peroxyacetic acid on mycelial growth in vitro and also in the field and greenhouse studies. Studies from the field show that products were effective in managing tan spot. Further analysis of the plant greenness showed that overall greenness was high in the treated plants than untreated across the cultivars. Grain yield was significantly higher for *Bacillus subtilis* QST713, and *Bacillus amyloquafasciens* D747 followed by *Streptomyces lydicus* Wyec 108. Our study indicated that biochemical and biological pesticides evaluated were effective in controlling tan spot and improving yield and plant vigor in spring wheat.

3.1 Introduction

Wheat production is affected by a number of constraints including pests and diseases. To overcome these, a combination of management practices including use of crop rotation, biocontrol fungicides, proper fertilization and use of resistant cultivars may be used to minimize the impact of pests in farming (Bockus, 1998; Jardine et al. 2000; Krupinsky et al. 2002, 2004; McMullen and Lamey, 1994; Turkington et al. 2003). Pesticides including herbicides, insecticides, and fungicides have contributed a great deal to substantial increases in yields and product quality (Osteen & Szmedra, 1989; Fernandez-Cornejo et al. 1998; Gardner et al. 2009, Fernandez-Cornejo et al. 2014). In the industrialized world, pest control is heavily dependent on the use of synthetic chemical inputs. These are not only very expensive but also are detrimental to the environment (Romeis & Meissle, 2006). The synthetic pesticides also present a plethora of risks to human health. The human and environmental risks can result from direct exposure of workers to pesticides or residues on consumables and movement of pesticides into the ground and surface water and into the food chain respectively (Council of Environmental Quality, 1993). It is also noted that the use of synthetic pesticides presents higher likelihoods of eliminating beneficial organisms (Hassan et al. 2014) which ultimately destabilizes the ecosystem (Schuler et al. 1998). The above detrimental effects from pesticides have partly led to a yearly increase in the usage of biochemical and microbial pesticides world over (Marketsandmarkets.com, 2018). Biopesticides are certain types of pesticides derived from natural materials like animals, plants, bacteria, and certain minerals. For example, canola oil and baking soda have pesticidal properties

and hence are considered biopesticides (Leahy et al. 2014). Biological control can also be defined as an "environmentally-friendly" strategy using living microorganisms or their derivatives to reduce a targeted pathogen. These have proven successful for example the use of dusting sulfur to prevent powdery mildew in late season cucurbit crops. Part of the biological control approach is the use of biocontrol agents (BCA) whose narrow spectrum provide an optimum efficacy without affecting non-targeted organisms (Alabouvette et al. 2006; Mendelsohn et al. 1994). The use of biochemical and microbial pesticides has been in existence for over 50 years but of recent, has started declining due to the emergence of resistance in the pest populations (Chandler et al. 2011). Plant extracts were likely the earliest agricultural biocontrols, as history records that nicotine was used to control plum beetles as early as the 17th century (BPIA, 2017). As of now, biopesticides are gaining popularity because of the sustainability benefits and the increasing concerns of the negative impact of residues arising from the overuse of synthetic chemical pesticides (Pertot et al. 2015). The biochemical and microbial pesticides are far less toxic with no to less residual effects as compared to the conventional pesticides hence less pesticide pollution problems associated with them. They (biochemical and microbial pesticides) also degrade faster and are not persistent in the environment. It is however advised that biochemical and microbial pesticides be used as a part of the Integrated Pest Management (IPM) program, not as "stand-alone" (Leahy et al. 2014). Use of agents like *Bacillus, Streptomyces*, and *Lysobacter* that can produce effective lytic enzymes or antibiotics confer protection to the plants against fungal diseases like tan spot (Luz et al. 2003; Whipps, 2001; Zhang et al. 2001; Luz et al. 1998; Gough & Ghazanfari, 1982). Most of these biopesticides are already marketed

commercially (Fravel, 2005). Biochemical and microbial pesticides have considerably less to none detrimental effects on the environment as compared to the synthetic counterparts (Romeis & Meissle, 2006; Lacey & Siegel, 2000). Related benefits from biopesticides like *Bacillus* and *pseudomonas flourecens* include promoting plant pigmentation processes (Mohamed & Gomma, 2012; Bertelsen & Neergaard, 2001). Antagonism to the pathogens is the mechanism used by most *Bacillus* strains to halt infection (Pérez-García et al. 2011). *Bacillus* strains are also known for their ability to produce biologically active molecules some of which are inhibitors of fungal pathogen growth (Schallmey and Ward, 2004). Other biochemical pesticides with recorded bactericidal, fungicidal and sporicidal properties are hydrogen peroxide and peracetic acid (Baldry, 1983)

Most biochemical and microbial pesticides are being used to manage diseases in crops like vegetables and fruits (ornamentals) (Doug and Ann, 2017) and are labelled for plants such as vegetables, fruits, nuts, ornamental trees, shrubs, flowering plants, houseplants, and tropical plants grown in and around home gardens or home greenhouses (Anonymous, 2018; Ockey et al. 2012). The labels also spell out many bacterial and fungal pathogens which the biochemical and microbial pesticides can control. However, no evaluations have been done on the efficacy of these products on field crops such as wheat for managing foliar diseases (US-EPA, 2005). One of the most pressing concerns in the synthetic fungicides arises from the prophylactic use of such fungicides which later puts at risk the ecosystem and the resultant development of resistance in a pest population (Van Emden & Service, 2004). Hence the objectives of this study were to 1) assess the efficacy of commercial biochemical and microbial pesticides in the management of tan

spot in wheat, 2) assess the efficacy of biochemical and microbial pesticides and other organic substrates in improving grain yield.

3.2 Materials and methods

A study to assess the efficacy of biochemical and microbial pesticides in the management of tan spot of wheat was carried out in the in vitro using Petri plates, in the greenhouse, and in the field plots at the Southeast Research Farm (SERF) organic and conventional fields.

3.2.1 In vitro mycelia growth inhibition assays

The in vitro experiments were conducted in the lab using biochemical and microbial pesticides -amended V8 PDA plates of size 100 x 15 mm (VWR® Radnor, PA). The in vitro efficacy of the biochemical and microbial pesticides was assessed based on the inhibition of mycelial growth measured by diameter (cm) of the ring formed by *Ptr* isolate growing on a treatment-amended plate in comparison with the positive control (pyraclostrobin) and the untreated check with no biochemical and microbial pesticides amendments. A *Pyrenophora tritici repentis* race 1 isolate "*Pti2*" was used for this study. This was used because it has already been characterized and its virulence is known (Lamari & Bernier, 1989b; Lamari et al. 2003). It presents symptoms of chlorosis and necrosis on the infected wheat leaves.

3.2.2 Culturing of the tan spot isolate

The isolate was recovered from -20 ^{o}C and a fresh culture was initiated on V8-PDA growth media (V8- 150 ml, PDA 7grams, Agar 10grams, $CaCO_3$ 3grams and 850

ml of distilled water) on Petri plates (Lamari & Bernier, 1989). The Petri plates containing the formerly frozen plugs were incubated under dark conditions at room temperature (21-23 ^{o}C) for 7 days. Four biochemical and microbial pesticides (Table 3.1) were used; *Bacillus amyloquafasciens* D747, *Streptomyces lydicus* Wyec 108, *Bacillus subtilis* QST713, hydrogen peroxide + peroxyacetic Acid, the synthetic positive check pyraclostrobin and the negative control check (untreated). V8- PDA media was autoclaved and left to cool in a water bath at 10 ^{o}C. The cooled media was aseptically amended with the products at recommended field concentrations of; *Bacillus amyloquafasciens* D747 19.6 ml/l, *Streptomyces lydicus* Wyec 108 at 4.6 ml/l, *Bacillus subtilis* QST713 at 39 ml/l, hydrogen peroxide + peroxyacetic Acid at 8.3 ml/l and finally pyraclostrobin at 2.3 ml/l poured onto the Petri plates (9cm diameter) from a running fume hood and left for 24 hours to solidify. Seven days old tan spot pure culture was used to make plugs using a flame-sterilized steel cork borer of 7/5mm outside inside diameter. A *Ptr* plug was placed on each of the biochemical and microbial pesticides -amended plates and also on the control (non-amended plates), hydrogen peroxide + peroxyacetic acid and a positive control of pyraclostrobin. The plates were transferred to the dark room maintained at 20-23 ^{o}C and left to incubate for 7 days. The design of this study was completely randomized and treatments were replicated five times. The experiment was repeated once.

3.2.3 Mycelia growth assessment

After 7 days of incubation in the dark, the plates were assessed for growth and measurements were taken on the diameter of the growth ring per plate per treatment using a Wescot® (R405-30cm, China) clear ruler. The diameter was taken from four diagonal

directions and later averaged. Data were collected for the second time at 12 days after incubation to ascertain further growth.

3.3 Greenhouse studies

A tan spot susceptible cultivar, "Select" was used for the greenhouse studies at the Plant Science greenhouse. Five seeds were planted in each cone and later thinned to four seedlings. Black "cone-tainers" of a cell diameter of 3.8cm and a depth of 20cm containing commercial soil "Pro-mix® BX mycorrhizae" (Greenhouse Megastore, Danville, IL) were used. The experiment was run for 8 weeks with inoculations and foliar biochemical and microbial pesticides application done at two different times on 3 and 6 weeks old plants, respectively. The products were applied at rates recommended by the manufacturers as per the label and MSDS (Table 3.1). All products were applied to the plant leaves except *Streptomyces lydicus* Wyec 108 which was applied in the soil. Percentage disease severity and number of lesions on the fully inoculated leaf were recorded at 7 and 14 days after inoculation on four plants in each "cone-tainer" and treatment set

3.3.1 Inoculum preparation and inoculation process

All plants in the greenhouse were inoculated with race 1 *Pti2* isolate of *Pyrenophora tritici repentis (Ptr)* known for producing chlorosis and necrosis on the infected leaves (Lamari & Bernier, 1989b; Lamari et al. 2003). The isolate was prepared as outlined in the in-vitro study. After 5 days of incubation under the dark condition, the V8-PDA plates had a fully grown *Ptr* fungus, which was matted down using a flame-sterilized test tube bottom and transferred to light for 24 hours followed by another round

of 24 hours of darkness at 16 ^{o}C to facilitate conidiation. Using a sterilized wire loop, the conidia was scrapped off and enumerated using a microscope. The conidial concentration was brought to 2000 spores/ml per inoculation. The diluted spores were transferred to a Preval® sprayer (Nakoma Products, LLC, Bridgeview, IL) and plants were inoculated until runoff on the leaves. The inoculated plants were transferred to the humidity chamber with misting for 10 seconds every after 12 minutes for a span of 24 hours to allow the 6-20 hours of leaf wetness that is favorable for tan spot fungus infection (De Wolf et al.1998; Lamari & Bernier, 1989). After 24 hours in the misting chamber, the rack containing inoculated plants was relocated to the greenhouse. Assessment of disease severity and lesion number was carried out at 7 and 14 days after inoculation (DAI) on the four plants in each "cone-tainer"/replicate per treatment set and this was later averaged.

3.4 Field studies

The field experiments were done at the Southeast Research farm located in Beresford, SD. Two spring wheat cultivars "Select" and "Brick" with varying susceptibilities to tan spot were planted in the spring of 2018 (April 23). Two separate fields were used, one organically certified field where only the organic biochemical and microbial pesticides were tested and another conventional field where a synthetic fungicide (pyraclostrobin) was included in as a positive check. The organic study had six treatments namely; *Streptomyces lydicus* Wyec 108, *Bacillus amyloquafasciens* D747, hydrogen peroxide + peroxyacetic acid, *Bacillus subtilis* QST713, azadirachtin, and a positive check pyraclostrobin. A negative control consisted of untreated wheat seedlings.

Treatments were applied at 4 leaf stage and a second application was after 10 days from the first treatment application (The first application of the microbial and biochemical products was 5/26/2018 and the next application was 6/20/2018). The products were applied using a pressurized hand operated boom sprayer at an average spray volume of 11 liters per hectare. Tan spot severity rating was conducted 10 and 15 days after the second application. Also, plant greenness for each plot was determined 15 days after the last treatment using a GreenSeeker® (Trimble Inc. Sunnyvale, CA). Plots were also rated for bacterial leaf streak to account for variation caused by this disease. Bacterial leaf streak severity was assessed at 15 and 25 days after product applications. Grain yield for all field plots was recorded at harvest.

3.5 Data analysis

Effect of biochemical and microbial pesticides on mycelial growth was computed with Analysis of Variance (ANOVA) after log transformation to homogenize the variance. Tan spot severity for the greenhouse and field studies was subjected to ANOVA for each assessment period. Treatment mean comparisons were performed using Fisher's protected Least Significant Difference (LSD) at ($P \leq 0.05$). All statistical analyses were conducted using R program 3.5.1 version (R Core Team, 2013).

3.6 Results

3.6.1 In vitro studies

From the in-vitro mycelia assay studies, mycelial growth was inhibited by three biopesticides *Bacillus subtilis*, *Bacillus amyloquafasciens*, and *Streptomyces lydicus* but

they were not significantly different from each other in the inhibition of mycelial growth. There was no significant effect of hydrogen peroxide + peroxyacetic acid on the *Ptr* mycelia growth (Table 3.2).

3.6.2 Greenhouse study

Two greenhouse runs were pooled together after conducting a homogeneity of variance test. To cater for the zero values, the data for disease severity and the number of lesions was log transformed. The results showed a significantly lower disease severity and number of lesions in the treated plants as compared to the untreated plants (Table 3.4). There was better disease control in plants treated with *Bacillus amyloquafasciens* D747, *Bacillus Subtilis* QST713, and *Streptomyces lydicus*. Hydrogen peroxide + peroxyacetic acid did not offer significant protection to the plants but had numerically lower disease in comparison with the check.

3.6.3 Field study

The field study comprised of the organic and conventional fields. Data from these two fields were separately analyzed. The results from the organic field indicated a significantly higher tan spot disease severity in the untreated plots both at 10 and 15 days after treatment. Products *Bacillus amyloquafasciens* D747, *Bacillus Subtilis* QST713, Hydrogen peroxide + peroxyacetic acid and lastly *Streptomyces lydicus* respectively had low tan spot severity (Table 3.5). The results from the conventional field had a significantly low tan spot disease severity amongst treatments at 10 and 15 days after treatment. The untreated check had significantly high disease severity compared to the treated plots but the other treatments were not significantly different from each other. At 15 days after planting, the check was significantly high in tan spot percent severity and

plants treated with pyraclostrobin had the lowest percent disease severity. Bacterial leaf streak severity was low under hydrogen peroxide + peroxyacetic acid, *Bacillus subtilis* QST 713 and pyraclostrobin treated plots.

3.7 Discussion

In the greenhouse, in vitro, and field studies, there was significant efficacy registered for biopesticides *Bacillus Subtilis* strain QST713, *Bacillus amyloquafasciens* D747. *Bacillus subtilis* strain QST713 is a widespread bacterium found in soil, water, and air. *Bacillus subtilis* strain QST713 is also known to control the growth of certain harmful bacteria and fungi. The mode of action of *Bacillus Subtilis* is said to be by competing for nutrients, growth sites on plants, and by directly colonizing and attaching to fungal pathogens (Bellet, 1998). Direct antagonism involves the production of several microbial metabolites among which lipopeptides play the major role (Pertot et al. 2015; Schallmey and Ward, 2004). Surfactin, in particular, is involved in the mechanism of resistance induction, hence there might have been some systemic acquired resistance in the plants treated with the *Bacillus* spp. There have been reports of induced resistance in plants treated with FZB24® strain of *Bacillus subtilis* in the control of powdery mildew in wheat (Kilian et al. 2000) and this could partly explain the low levels of disease severity and inhibited mycelia growth in the greenhouse, field and in vitro studies respectively for the treatments *Bacillus subtilis* QST713 and *Bacillus amyloquafasciens* D747. The suppression of mycelial growth and tan spot disease severity by *Streptomyces lydicus* might indicate the presence of some bioactive compounds with an antifungal activity on *Pyrenophora tritici repentis.* The previous ability of *Bacillus* strains to control fungal

plant diseases has been attributed to iturins and fengycins (Ongena and Jacques, 2008; Romero et al. 2007; Arrebola et al. 2010). Fengycins show strong fungitoxic activity to filamentous fungi (Ongena and Jacques, 2008). Previous studies have reported that *Streptomyces* is used as fungicides for the control of rice blast, for instance, Blasticidin-S (isolated from *S. griseochromogenes*) and Kasugamycin are some of the common antibiotics produced by *Streptomyces* (Fukunaga et al. 1955; Takeuchi et al. 1958; Tapadar and Jha, 2013). *Mildiomycin* produced by the soil actinomycete *Streptoverticillium rimofaciens* strain B- 98891 (Harada and Kishi, 1978; Om et al. 1984) is specifically active against the pathogens that cause powdery mildew and is applied as a foliar spray. However, foliar application of *Streptomyces lydicus* D747 did not offer any protection to the plants (data not shown) even when some studies have reported successful control of rice blast through foliar spray (Yang et al. 2008; Laborte et al. 2012; Gopalakrishnan et al. 2014). All these successful biopesticides disease management are in agreement with the results of this study. There was a complete stoppage of *Ptr* mycelial growth in Petri plates amended with pyraclostrobin. This is because of the effect of strobilurins on electron transport in the mitochondria where they bind to the ubihydroquinone reduction site, the Q_o-site of complex bc1, thus inhibiting electron transfer between cytochrome b and cytochrome c1 in the respiratory chain. This in turn severely reduces the aerobic ATP production, thereby inhibiting the growth of the fungus (Godwin et al. 1994; Shirane et al. 1994; Leinhos et al. 1997; Sauter et al. 1999; Bartlett et al. 2002). Also, the conventional field and greenhouse studies registered significantly less tan spot disease severity in plants treated with pyraclostrobin fungicide which verifies previous reports with pyraclostrobin that pointed out its successful

management of tan spot in wheat (Wegulo, 2006, Turkington et al. 2003). Related benefits of a yield increase from using foliar fungicides have been registered before in strobilurins (Wegulo, 2006; Turkington et al. 2003; Bertelsen & Neergaard, 2001) and this could explain the higher yields in the conventional plots where pyraclostrobin was applied.

Plots treated with azadirachtin had no significant tan spot disease control in the organic field at 10 days after treatment application, but a less numerical tan spot disease percentage at 15 days after treatment application was registered. Also, plants treated with azadirachtin were found to be less green compared to other treatments. This is because the active ingredient azadirachtin acts majorly as an insecticide not a pathogen control agent (Immaraju, 1998) and the less numerical disease percentage could have been because of the allelochemicals with the antifungal properties that azadirachtin is said to possess (Koul et al. 1994). Overall, plant vigor assessed based on greenness was high in plants treated with biopesticides and pyraclostrobin even though they were not significantly different amongst themselves but were significantly greener than the check plots. The improved plant greenness can in part be attributed to the considerable protection against foliar diseases by the microbial pesticides, soil factors, and cultivar attributes. However, a study by Bertelsen & Neergaard, (2001) showed an increased and prolonged plant greenness on plants that had been treated with azoxystrobin. Since azoxystrobin and pyraclostrobin are both strobilurins, this might explain in part the high greenness recorded in plots treated with pyraclostrobin compared to the check. Also in a study by Mohamed and Gomma (2012) involving *Bacillus subtilis* and *Pseudomonas flourescens* there was increased greenness in the plants that were treated. The increased

plant growth promotion and health marked by greenness in the treated plots can further be supported by (Gopalakrishnan et al. 2014) whereby in their study, *Streptomyces* strains significantly enhanced tiller and panicle numbers, stover and grain yields, dry matter, root length, volume, and dry weight, compared with the control for rice. There's also a high likelihood of plant growth promotion by the microbial pesticides especially *Bacillus* and *Streptomyces* attained by modulation of plant development through the production of phytohormones (Tsavkelova et al. 2006). The used microbial pesticides were most probably able to produce auxins that ultimately stimulated root proliferation and nutrient uptake (Spaepen et al. 2007). *Bacillus amyloquafasciens* biosynthesizes indole-3-acetic acid (IAA) which is responsible for plant growth promotion (Idris et al. 2007) and this can also explain the recorded high plant greenness. In addition, *Bacillus subtilis* produces cytokinin which has a beneficial effect on plant growth (Arkhipova et al. 2007 and Ortı´z-Castro et al. 2008). All these studies match the results reported by the high green intensity in plants treated with microbial pesticides strains of *Bacillus subtilis, Bacillus amyloquafasciens* and *Streptomyces lydicus* at the Southeast research farm organic and conventional fields.

There were general small percentages in tan spot disease severity ratings and yields in the field study. This is partly because of the impact of bacterial leaf streak and weeds which affected all our study plots later in the season. The infection with bacterial leaf streak was true for most parts of South Dakota in 2018 (Byamukama, 2018). The field study was also conducted from one location and not repeated hence there is a need for getting data on the effect of biopesticides across varied environments. The small but significantly different tan spot percentage disease severity in the field plots was because

of the infection from inoculum blown by the wind from the adjacent fields because plants were not planted on wheat stubble. There's a need to evaluate these biopesticides products under no-till practices. This is because biopesticides are often strongly influenced by environmental conditions (e.g. temperature, humidity, pH, etc.), which can result in variable efficacy (Gaultier, 2009). In addition, the biopesticides have limited selectivity a reason this study could not conclusively verify if there was a biopesticides effect on Bacterial Leaf Streak (BLS) in the field study even when our results indicated varying levels of severity for BLS among treatments. The low BLS severity in plots treated with pyraclostrobin is most likely due to natural variation, not the effect of the pesticide in addition to pyraclostrobin being a fungicide with no reported control effect on bacteria.

Results from the organic field indicated that yield was significantly high in plots treated with *Bacillus subtilis* QST713 and the next high yields were in plots treated with *Bacillus amyloquafasciens* D747, hydrogen peroxide + peroxyacetic acid, azadirachtin, *Streptomyces lydicus* Wyec 108 respectively even when they were not significantly different from each other. The check had the least kilograms per hectare of grain yield. In the conventional field, yield was significantly high in the plots treated with pyraclostrobin followed by *Bacillus subtilis* QST713, *Bacillus amyloquafasciens* D747, *Streptomyces lydicus* Wyec 108 which were not significantly different from each other. The smallest yield was in hydrogen peroxide+ peroxyacetic acid and check. The observed yield increase can be attributed to the fact that the biopesticides products and other foliar fungicides are able to offer protection to the flag leaf which is important for grain filling (Fernandez et al. 2014, Turkington et al. 2004 & 2015, Poole & Arnaudin, 2014).

Overall, this study reveals hope for microbial pesticides to be used in the organic tan spot disease management in wheat. This is based on the in vitro, greenhouse and the field results that this study has reported.

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Table 3.1: Treatments used in the greenhouse and Southeast Research Farm (SERF) field study to assess the efficacy of microbial and biochemical pesticides in managing tan spot

Common name	**Active ingredient**	**Rate**
Actinovate ® Lawn and Garden	*Streptomyces lydicus* Wyec 108	4.6 ml /l
Monterey ® complete disease control	*Bacillus amyloliquefaciens* strain D747	19.6 ml/l
Serenade ® garden disease control	*Bacillus subtilis* QST713	39 ml/l
Biosafe ®disease control-concentrate and RTU	Hydrogen peroxide + peroxyacetic acid.	8.3 ml/l
Safer ® Bio Neem	Azadirachtin	29 ml/l
Headline ® F3	Pyraclostrobin[a]	2.3 ml/1

[a]Pyraclostrobin was used as a positive check in the conventional block,

Table 3.2: Effect of microbial and biochemical pesticides on the growth of *Pyrenophora tritici repentis* (diameter in centimeters) in vitro recorded at 7 and 14 days after incubation

Days after incubation, Treatment	**Length (cm, Log +10)**
7 DA1[a]	
Check	1.1 a
Hydrogen peroxide + peroxyacetic acid	1.1 a
Pyraclostrobin	1 c
Bacillus amyloquafasciens D747	1 c
Bacillus subtilis QST713	1 c
Streptomyces lydicus	1 c
14 DAI	
Check	1.3 a
Hydrogen peroxide + peroxyacetic acid	1.12 b
Pyraclostrobin	1 c
Bacillus amyloquafasciens D7474	1 c
Bacillus subtilis QST713	1 c
Streptomyces lydicus	1 c

Values are least sqaured means of 5 replications. Variables with the same letters are not significantly different from each other according to Fishers Least Significant Difference Test, ($P \leq 0.05$)

[a]DAI Days After Incubation

Table 3.3: Effect of microbial and biochemical pesticides on tan spot disease severity and lesion numbers for plants inoculated at 3 and 6 weeks after planting in the greenhouse

Weeks after planting, Treatment	Number of Lesions [b](log+10)		% Disease severity(log+10)	
3 Weeks				
Check	1.66	a	1.44	a
Streptomyces lydicus Wyec 108	1.39	b	1.21	b
Hydrogen peroxide + peroxyacetic acid	1.26	c	1.11	c
Bacillus subtilis QST713	1.03	d	1.01	d
Bacillus amyloquafasciens D747	1.03	d	1.00	d
Pyraclostrobin	1.00	d	1.00	d
6 weeks				
Check	1.71	a	1.83	a
Hydrogen peroxide +peroxyacetic acid	1.49	b	1.45	b
Streptomyces lydicus Wyec 108	1.35	c	1.20	c
Bacillus subtilis QST713	1.08	d	1.03	d
Bacillus amyloquafasciens D747	1.05	de	1.02	d
Pyraclostrobin	1.01	e	1.00	d

Values are 64 replications from the two runs. Treatments with the same letters are not statistically different ($P \leq 0.05$)

[b] Disease severity and lesions are log-transformed values

Table 3.4: Effect of microbial and biochemical pesticides on tan spot severity in the organic field block at Southeast Research Farm.

Treatment	Tan spot severity (%)			
	10 DAT[a]		15 DAT	
Check	12	a	24	a
Azadirachtin	5	b	7	b
Bacillus subtilis QST713	2	c	5	b
Hydrogen peroxide+ peroxyacetic acid	2	c	11	b
Bacillus amyloquafasciens D747	1	c	4	b
Streptomyces lydicus Wyec 108	1	c	6	b

Values are least sqaured means of 8 replications. Variables with the same letters are not significantly different from each other according to Fishers Least Significant Difference Test, (P value ≤ 0.05)

[a]DAT Days After Treatment

Table 3.5: Effect of microbial and biochemical pesticides on tan spot severity in the conventional field at Southeast Research Farm.

Treatment	**Disease severity (%)**	
	10 DAT[a]	**15 DAT**
Check	6.6 a	14 a
Bacillus amyloquafasciens D747	2.1 b	6 bc
Hydrogen Peroxide+ Peroxyacetic acid	1.5 b	8 ab
Bacillus subtilis QST713	1.3 b	8 ab
Streptomyces lydicus WYEC 108	0.8 b	8 ab
Pyraclostrobin	0.7 b	1 c

Values are least squared means of 8 replications. Variables with the same letters are not significantly different from each other according to Fishers Least Significant Difference Test, ($P \leq 0.05$)
[a]DAT Days After Treatment

Table 3.6: Effect of microbial and biochemical pesticides on plant greenness in the conventional field at the Southeast Research Farm across the two cultivars

Cultivar, Treatment	**Greenness**
Select	
Bacillus amyloquafasciens D747	0.7 a
Bacillus subtilis QST713	0.7 a
Hydrogen peroxide+ peroxyacetic acid	0.7 a
Pyraclostrobin	0.7 a
Streptomyces lydicus Wyec 108	0.7 a
Check	0.3 b
Brick	
Bacillus amyloquafasciens D747	0.6 a
Streptomyces lydicus Wyec 108	0.6 a
Hydrogen peroxide+ peroxyacetic acid	0.6 a
Bacillus subtilis QST713	0.5 a
Pyraclostrobin	0.5 a
Check	0.2 b

Values are least sqaured means of 4 replications for each cultivar.
Variables with the same letters are not significantly different from each other according to Fishers Least Significant Difference Test, ($P \leq 0.05$)

Table 3.7: Effect of microbial and biochemical pesticides on plant greenness in the organic field at Southeast Research Farm across the two cultivars

Cultivar, Treatment	Greenness	
Select		
Bacillus amyloquafasciens D747	0.57	a
Streptomyces lydicus Wyec 108	0.56	a
Bacillus subtilis QST713	0.56	a
Hydrogen peroxide+ peroxyacetic acid	0.54	a
Azadirachtin	0.52	ab
Check	0.4	b
Brick		
Streptomyces lydicus Wyec 108	0.6	a
Hydrogen peroxide+ peroxyacetic acid	0.6	a
Bacillus amyloquafasciens D747	0.6	a
Bacillus subtilis QST713	0.5	ab
Azadirachtin	0.5	ab
Check	0.4	b

Values are least squared means of 4 replications for each cultivar. Variables with the same letters are not significantly different from each other according to Fishers Least Significant Difference Test, P value ≤ 0.05

Table 3.8: Effect of microbial and biochemical pesticides on Bacterial leaf streak (BLS) in the conventional field at the Southeast Research Farm

Days after Treatment, Treatment	BLS disease severity (%)	
15 DAT[a]		
check	21.4	a
Bacillus amyloquafasciens D747	18.4	ab
Hydrogen peroxide + peroxyacetic acid	13.3	ab
Pyraclostrobin	11.6	ab
Streptomyces lydicus Wyec 108	11.0	b
Bacillus subtilis QST713	10.6	b
25 DAT		
check	74.4	a
Streptomyces lydicus Wyec 108	52.1	ab
Bacillus amyloquafasciens D747	51.8	ab
Bacillus subtilis QST713	46.8	b
Hydrogen peroxide +peroxyacetic acid	37.1	b
Pyraclostrobin	36.5	b

Values are least squared means of 8 replications. Variables with the same letters are not significantly different from each other according to Fishers Least Significant Difference Test, ($P \leq 0.05$)

[a]DAT Days After Treatment

Table 3.9*:* Effect of biochemical and microbial pesticides on Bacterial Leaf Streak in the organic field at the Southeast Research Farm

DAT[a], Treatment	BLS[b] disease severity(%)	
15 DAT		
Check	39	a
Streptomyces lydicus Wyec 108	20	ab
Bacillus amyloquafasciens D747	11	b
Azadirachtin	10	b
Bacillus subtilis QST713	7	b
Hydrogen peroxide + peroxyacetic acid	5	b
25 DAT		
Check	70	a
Azadirachtin	70	a
Hydrogen peroxide + peroxyacetic acid	64	a
Bacillus amyloquafasciens D747	64	a
Streptomyces lydicus Wyec 108	63	a
Bacillus subtilis QST713	63	a

Values are least squared means of 8 replications. Variables with the same letters are not significantly different from each other according to Fishers Least Significant Difference Test, ($P \leq 0.05$)
[a]DAT Days after treatment with the biopesticides
[b]BLS Bacterial leaf streak

Table 3.10: Effect of microbial and biochemical pesticides on yield assessed in the conventional field at Southeast Research Farm.

Treatment	Yield (kg/ha)	
Pyraclostrobin	1789	a
Bacillus subtilis QST713	1362	ab
Bacillus amyloquafasciens D747	1315	ab
Streptomyces lydicus Wyec 108	1303	ab
Hydrogen peroxide + peroxyacetic acid	855	b
Check	709	b

Values are least squared means of 8 replications. Variables with the same letters are not significantly different from each other according to Fishers Least Significant Difference Test ($P \leq 0.05$)

Table 3.11: Effect of microbial and biochemical pesticides on yield assessed in the organic field at Southeast Research Farm.

Treatment	**Yield (kg/ha)**	
Bacillus subtilis QST713	2151	a
Bacillus amyloquafasciens D747	2009	ab
Hydrogen peroxide + peroxyacetic	1970	ab
Azadirachtin	1835	ab
Streptomyces lydicus Wyec 108	1722	ab
Check	1117	b

Values are least squared means of 8 replications. Variables with the same letters are not significantly different from each other according to Fishers Least Significant Difference Test, ($P \leq 0.05$)

CHAPTER FOUR

4.0 Investigating fungicide resistance in *Pyrenophora tritici-repentis*

Abstract

Fungicides are effective in managing many crop fungal diseases. However, repeated exposure of some fungicide active ingredients can increase the risk of developing resistance in the target population. In this study, 90 *Pyrenophora tritici-repentis* isolated collected in 2013, 2014, 2016 and 2017 were tested for sensitivity/insensitivity to three commonly applied fungicides in South Dakota. Fungicides with active ingredients picoxystrobin, prothioconazole + tebuconazole and azoxystrobin + propiconazole were used at three different dosages of the half, full, and double the field recommended application rates. Assays involved mycelia and spore germination testing on V8PDA, potato dextrose broth, and water agar respectively. Initial results from the mycelia assays using microtiter plate method showed thirty out of ninety isolates could grow in picoxystrobin half and full dosages amended broth. Five out of the thirty isolates were further tested on V8PDA fungicide amended plates using mycelia plugs and growth was noticed on picoxystrobin whereas prothioconazole + tebuconazole and azoxystrobin + propiconazole indicated 100 % inhibition of growth. Assays of five isolates on water agar using conidia spores revealed germination of the five isolates on all the three fungicides with a higher growth percentage recorded in picoxystrobin. Lower percentage spore germination was recorded in prothioconazole + tebuconazole and azoxystrobin + propiconazole which were not significantly different. Further Studies involving the use of Salicylhydroxamic acid (SHAM) were conducted on four out of the five isolates and the findings of this study indicate complete stoppage of spore germination. Findings of this

study indicate some level of insensitivity to picoxystrobin without SHAM and complete stoppage of germination with SHAM. Based on previous studies involving the use of SHAM to curtail the impact of alternative oxidase on strobilurins, we found no insensitivity amongst the isolates tested.

4.1 Introduction

Chemical approaches to disease plant disease management are descriptive of modern agriculture because of the benefits in increasing crop yields and quality (Lucas et al. 2015). Fungicides have been used in agriculture for a long time but there was no evidence of resistance by that time (Horsfall, 1945). There are a number of successful disease management at low-level fungicide dosages (Russell, 2005) which partly explains why the use of fungicides remains essential in agriculture production in terms of maintaining healthy, reliable and high yielding crops. Fungicide application is an indispensable approach in the management of diseases like Fusarium head blight, tan spot and many other fungal diseases (Bai et al. 2003; Mesterházy, 2003; Dill-Macky et al. 2000; Ogundana and Denis, 1981; Plumbley, 1985). Fungicides with different modes of actions are used to manage wheat diseases. The commonly used classes in wheat disease management are strobilurins, triazoles, Succinate Dehydrogenase Inhibitors (SDHIs) and the Methyl benzimidazole-2-yl Carbamate (MBC). The wide use of especially triazoles in wheat disease management is partly because they are more effective than other active ingredients (Paul et al. 2008; Dubos et al.2011 and 2013& Sun et al. 2014) and they act by affecting ergosterol biosynthesis (Paul et al. 2008). Other fungicides with multiple modes of actions like metconazole + pyraclostrobin, prothioconazole + trifloxystrobin,

propiconazole + azoxystrobin, propiconazole + azoxystrobin, propiconazole + trifloxystrobin and tebuconazole + trifloxystrobin are also in use in the state of South Dakota (Fanning et al. 2012). The above and other modes of actions are the currently employed mechanisms in the chemical control of plant diseases in the US. There are reports of declining triazole sensitivity for example in Germany, a number of fungicide resistance cases were reported in 1987 (Klix et al. 2007; Krupinsky, 1982). As a result of the massive and injudicious use of fungicides, there are reports of declining sensitivities in wheat pathogens. The use of fungicides with similar modes of action for a long time and repeatedly is the principal cause of resistance (Deising et al. 2002). Both qualitative resistance which results from mutations in genes encoding fungicide targets (Ishi et al. 2001) and the polygenic quantitative resistance that leads to cross-resistance have been reported (Brent & Hollomon, 1998). Quantitative resistance can also be triggered by the use of alternative metabolic pathways hence it is highly desired to know the mode of resistance of the target fungal pathogen before developing any anti-resistance strategies (Brent et al. 1998). In South Dakota, fungicides in the class of strobilurins and azoles such as azoxystrobin, pyraclostrobin and cyproconazole, metconazole, propiconazole, prothioconazole, tebuconazole, and prothioconazole + tebuconazole among others are mostly used in managing common fungal wheat diseases including tan spot, stripe rust, and Fusarium head blight (Fanning et al. 2012). Strobilurins are known to have a higher risk of resistance (Vincelli, 2002).

In 2003, the first *Pyrenophora tritici repentis (Ptr)* isolates with reduced sensitivity to strobilurins were reported (FRAC, 2002). By 2004, reduced sensitivity in *Ptr* was observed in field populations, leading to the detection of the first quinone outside

inhibitor (QoI) reduced sensitivity in *Ptr* in middle regions of Sweden (Sierotzki et al. 2007). It is therefore important to screen *Ptr* isolates for QoI and azole sensitivity in order to develop tan spot management plans that are sustainable. Therefore, the objective of this study was to determine the sensitivity of tan spot pathogen isolates to the commonly used fungicides in the Northern Great Plains.

4.2 Materials and methods

In this study, 90 isolates from wheat leaves collected from Montana, Kansas, North Dakota, Oklahoma, and South Dakota were tested for fungicide sensitivity. The assays were conducted in the microtiter plates and Petri plates (Size 100 mm x 20 mm). This was done using fresh mycelial plugs and spores scrapped from newly cultured *Ptr* isolates.

Tan spot symptomatic leaves were collected from wheat fields across five states in the Northern Great Plains (NGP) region. For each state, three counties with high wheat acreage were selected arbitrarily and from each county, five wheat fields were arbitrarily selected. In each field, four leaves with tan spot symptoms were picked from four plants at each of five stops (a total of 20 leaves per field) within a field in a "W" pattern. The leaves were placed in a paper bag and labeled accordingly and shipped to South Dakota State University. The leaves were kept at 4 ^{o}C until isolations were done.

4.2.1 Isolation of *Ptr* from leaf tissues for sensitivity studies

Symptomatic leaf samples were cut into small pieces of approximately 1 cm in length. About 5-7 pieces of leaf tissue with symptoms from each of 20 leaves from the field were surface-sterilized in 1% bleach for 30 seconds and rinsed in sterile distilled

water for 1 min. The surface-sterilized leaf tissue was placed on glass Petri dishes with two layered wetted filter paper (Whatman3, Sigma-Aldrich Corp. St. Louis, MO USA). The leaf pieces were incubated at 20-23 ^{o}C with a 24-hour dark period followed by another round of 24 hours of white fluorescent light before examination for sporulation. The leaf tissues were later examined under a stereoscope. Upon observation of conidia, a flame sterilized needle was cooled down and used to excise the conidia from the leaf tissue and transferred the V8 PDA media. Multiple isolations were made from each of the 5-7 tissues on the plate and were later transferred to the dark at room temperature (20-23 ^{o}C). One day later, further isolations from the edges of the growing fungus on V8 PDA media were done to get a pure culture and these were left to grow for a period of 5-7 days under dark at room temperature in the dark. Using a flame-sterilized cork borer several plugs were made and left to dry overnight and were used the next day for the fungicide sensitivity study or inserted in sterile vials which were kept at freezer at -20 ^{o}C for future use.

4.2.2 Microtiter plate assays

Using a 96-well microtiter plate, *Ptr* plugs (0.2-0.3 cm) from different isolates were cultured on a fungicide amended potato dextrose broth(PDB) in each well Potato dextrose broth was prepared by suspending 24g of the powder in 1000 ml distilled water and autoclaving the solution for 30 minutes. The PDB was left to cool down in a water bath at 23 ^{o}C for 1 hour before mixing it with the fungicides and pouring in the microtiter wells. Three fungicides with different modes of action were used. These were picoxystrobin, prothioconazole + tebuconazole and a mixed mode of action fungicide with active ingredients azoxystrobin + propiconazole at label rates of 1.7 ml/l, 2.75 ml

and 4.46 ml/ l, respectively (Table 4.2). The fungicide concentrations were determined from the recommended field application label at the half and full rates (United States National Plant Board, 2001). The fungicide products were mixed with potato dextrose broth and using a pipette, 250µl of the broth + fungicide or broth alone were transferred to the wells of the microtiter plate for the half dose, full, double fungicide rates. Isolates in form of mycelial plugs of 0.3cm diameter were dipped upside down into the fungicide amended broth or PDB alone making sure that they were in full contact with the solution. The growth of *Ptr* was visually assessed based on visible mycelial growth in the wells. To ascertain a complete insensitivity to the fungicide, the isolates were further grown in PDB amended with twice the recommended fungicide rates. The microtiter plate with all the treatments was covered with a parafilm and wrapped in aluminum foil to create dark conditions. The plates were left to incubate at room temperature of about 20-23 ^{o}C. Assessment of the microtiter plates was conducted five days after incubation since in the first three days, there was limited mycelial growth in the wells. The microtiter plate was examined with the aid of a stereoscope (Carl Zeiss Inc., Thornwood, NY) to visually assess mycelial growth. Wells, with hyphal masses in the presence of a fungicide, were regarded as insensitive and those where the inserted plug had no single mycelia growth were recorded as sensitive to the fungicide. These were compared with the non-fungicide amended wells which had a prolific mycelial growth. Three replications were used for each isolate and fungicide concentration. The well was the experimental unit and a randomized complete design was used.

4.2.3 Petri plate mycelial growth assays

The isolates that had shown fungicide insensitivity from the microtiter plate method were further confirmed using a Petri plate method. This was done using fungicide amended V8 PDA plates and five out of the thirty isolates were further tested (Tables 4.1 and 4.3). These were selected randomly selected from the 30 isolates using the RAND function from Microsoft Excel 2007 (Microsoft Corp. Redmond, WA). The *Ptr* growth media V8 PDA (V8 Juice 150 ml; $CaCO_3$ 3grams, Potato Dextrose Agar 10 grams; agar 10 grams; distilled water 850 ml) was prepared and the mixture autoclaved at chamber temperatures of 121 ^oC for one hour. The media was thereafter cooled down in a water bath at 40 ^oC for 15 minutes. When it was cool enough, one liter of the media was poured in a beaker using a graduated cylinder and the fungicide treatments were added and mixed well. Fungicides at a rate of 1.7 ml/l, 2.75 ml/l and 4.46ml/l for picoxystrobin, prothioconazole + tebuconazole and a mixed mode of action fungicide with active ingredients azoxystrobin + propiconazole respectively were added to the Petri plates and labeled accordingly. The fungicide-media mixture was poured on sterile Petri plates and left to solidify from a running laminar flow hood for 24 hours. The rates were further halved and doubled. Non-fungicide amended plates were also prepared as the control checks. The recovered *Ptr* plugs were placed in the center of the solidified fungicide and V8-PDA mixture plates and the control (V8-PDA with the plug only) and wrapped it in aluminum foil to create darkness and left to grow at room temperatures (22-23 ^oC). After 5 days of incubation under the dark condition, the V8-PDA plates were assessed. The second assessment of the growth of the cultures was done 10 days after incubation. In this case, the Petri plate was the experimental unit and the fungicides and the isolates were the

treatments. Three replicates per treatments were used and the plates were arranged in a completely randomized design. Growth was assessed by measuring the radial growth of the isolates on the plates using a ruler (R405-12in China). Four diagonal measurements were done and the average recorded. These were contrasted with the control plates for each of the isolates and fungicides for the 5 and 10 days old cultures. Isolates that showed radial growth on the Petri plate amended with a fungicide were regarded as insensitive and these were compared to the non-fungicide amended plate

4.2.4 Petri plate spore assays

Technical grade fungicides with picoxystrobin, azoxystrobin + propiconazole, tebuconazole + prothioconazole active ingredients were used to make solutions based on field recommended application rates. The sensitivity of *Pyrenophora tritici repentis* isolates was tested using spore germination assays on water agar. Isolates were revived from the freezer (-80 ^{o}C) and grown to full maturity for 7 days at room temperature in the dark to allow sporulation. After sporulation, about 1 ml of sterile distilled water was added to the external mycelia ring of the fungal colony where the spores were located, and the surface of the colony was gently rubbed using a metal loop to scrap off the spores. The spores in glass flasks were maintained on ice to avoid germination. Spore suspensions for each isolate were prepared in a 0.05% Tween solution (J.T. Baker. Phillipsburg, NJ), at 4000 spores ml^{-1}. Three hundred microliters of spore suspension for individual isolates were pipetted onto fungicide-amended water agar media (how was water agar prepared?) and spread using a sterile glass spreader (Carolina® Biologicals, Burlington, NC). After incubation in light at 22-23 °C for 18 hours, percentage spore germination was determined based on microscopic examination of a minimum of 50

spores for each isolate and fungicide concentration. A spore was considered non-germinated if the germ tube was shorter than the conidia itself or had no germ tube at all. The percentage germination was evaluated for each isolate and for each fungicide concentration treatment.

All the germinations were adjusted relative to the non-fungicide amended control plates for each of the isolates. There were two replicates of each of the five isolates for each of the three fungicides at three concentrations plus the non-fungicide amended control for each of the isolates. The microtiter wells were the experimental units and the study employed a complete randomized design for the spore and mycelia germination studies.

4.2.5 Petri plate salicylhydroxamic acid (SHAM) amended spore and mycelial assays

Studies involving the amendment of SHAM (Alfa Aesar, Ward Hill, MA) in the Petri plate assays were conducted on four isolates whereby a plug was placed onto a plate amended with SHAM + picoxystrobin. To prepare SHAM, a stock solution (100,000 mg/ml) was prepared by adding 0.1g of SHAM to 1ml of 99.9% methanol and the mixture was warmed at 37 ^{o}C for 8-10 minutes to dissolve the SHAM as described by (Pasche et al. 2007). The same SHAM concentration was used for the spore assays where SHAM was mixed with 2% water agar and the half and full recommended picoxystrobin rates. The plates were left to incubate under light for 16 hours and were later evaluated on the microscope. Spores with germ tubes shorter than the conidia and those without germ tubes were considered non-germinated.

4.3 Results

4.3.1 Microtiter plate mycelia plug assay results

Out of the ninety total Ptr isolates assessed on the microtiter plates, thirty plugs (Table 4.1) were still able to grow in the presence of half and full dosage of picoxystrobin. Other fungicides had no mycelia growth in the microtiter assays.

4.3.2 Petri plate mycelia assay results

Out of the thirty isolates that showed some level of insensitivity under the microtiter plate method, five of these had some growths in the presence of the half, full and double the recommended dose for picoxystrobin (Table 4.4). There was complete inhibition of mycelial growth in prothioconazole + tebuconazole and azoxystrobin + propiconazole (data not shown). The isolates were not statistically different from each other in sensitivity to picoxystrobin (Table 4.4). There was, however, a significant fungicide and days after incubation interaction for the isolates (Table 4.4). There was no mycelial growth in the double dose of picoxystrobin at 7 days after incubation but there was a measurable growth 12 days after incubation (Table 4.5)

4.3.2.1 SHAM amended Petri plate mycelia assays

Studies involving Salicylhydroxamic acid (SHAM) were conducted on four of the five tested isolates using picoxystrobin. The results indicated complete stoppage of mycelial growth in the treated plates. SHAM was also found to inhibit mycelia growth as was observed by reduced radial growth in the plates amended with SHAM only compared to the non-SHAM and non-fungicide, check plates.

4.3.3 Petri plate conidia spore assay

In the conidial assay, the isolate-fungicide treatment interaction was significant (Table 4.6). Percentage spore germination was overall low in isolates under azoxystrobin + propiconazole double rate but was high in isolates under picoxystrobin half, full and double dosages (Table 4.7). Different isolates had different sensitivity levels to the active ingredients and dosages for both fungicides.

4.3.3.1 Salicylhydroxamic acid (SHAM) amended Petri plate conidia spore assays

Four of the five isolates tested on Petri plates were sensitive to the full and half rates of picoxystrobin with SHAM amendment since there was no spore germination observed (Table 4.8). There was a significant difference between the isolates plated on water agar amended with SHAM and picoxystrobin to those without SHAM (Table 4.9). There was no single conidial germination in the plates where SHAM + picoxystrobin was used.

4.4 Discussion

Preliminary results from the microtiter and Petri plate studies indicated that using mycelia growth assays, 30 out of the 90 isolates tested were insensitive to picoxystrobin fungicide. There was complete inhibition of mycelial growth by prothioconazole + tebuconazole and azoxystrobin + propiconazole, the triazole fungicides. The mycelia inhibitory results using microtiter plates and V8PDA by prothioconazole + tebuconazole and azoxystrobin + propiconazole fungicide amended Petri plates in this study are in agreement with a study by Taskeen-Un- Nisa et al (2011) where hexaconazole inhabited mycelial growth in *Fusarium oxysporum*; epoxiconazole also inhabited mycelial growth

in *Gibberella zeae* isolates (Klix et al. 2006) which are both ascomycetes just like *Pyrenophora tritici repentis*. Triazoles are inhibitors of the ergosterol biosynthesis pathway (Siegel, 1981). The fungicides that inhibit biosynthesis of ergosterol will not necessarily inhibit spore germination (Ramirez et al. 2004). This is in agreement with the results obtained for fungicides, prothioconazole + tebuconazole and azoxystrobin + propiconazole in the study using *Pyrenophora tritici repentis* mycelial plugs in the microtiter plate wells and Petri plates as experimental units. On the other hand, there was mycelial growth in some isolates tested using picoxystrobin amended microtiter plates and Petri plates without SHAM. This is because strobilurins act as preventatives by effectively killing germinating spores which are devoid in the mycelia (Vincel P, 2002, Olaya et al. 1998). Also, the germinating spore that starts the infection process on the plant is more sensitive to QoI fungicides than is the mycelium. Several other studies have been reported where mycelial stages of pathogens were not controlled by strobilurins (Mizutani et al. 1996 and Hayashi et al. 1996). Since strobilurins/QoI fungicides target spore germination to achieve efficacy (Olaya et al. 998), assessment of spore germination is the best approach for sensitivity studies in chemicals that use this mode of action. In this study, the spore assays showed five tested *Pyrenophora tritici repentis* isolates had some spore germination in picoxystrobin, prothioconazole + tebuconazole and azoxystrobin + propiconazole fungicides with the highest percentages of germination in picoxystrobin (Table 4.7). The recorded spore germination in picoxystrobin fungicide amended water agar is a signal to a possible insensitivity but this cannot be fully verified since some fungal pathogens have been found to use the alternative oxidative pathway (AOX) allowing ATP synthesis to continue by evading the cytochrome b site of

QoI/strobilurins action (Vanlerberghe & McIntosh, 1997; Patel et al. 2012). This was later ruled out by amending plates with both picoxystrobin and SHAM using four isolates which later proved that all isolates were sensitive to the fungicide. Also, mycelia assay with picoxystrobin + SHAM indicated a sensitive response as evidenced by no single mycelia growth in SHAM amended plates (data not shown). The use of SHAM to avoid the effect of AOX on strobilurins has been reported in previous studies whereby Salicylhydroxamic acid (SHAM) was incorporated to avoid confounding results in the laboratory assessment of *Ptr* (Jin et al. 2009). Similar effects of SHAM inhibiting the activity of the alternative oxidase(AOX) were reported in studies by (Maclean et al. 2017; Sierotzki et al. 2007; Vincelli and Dixon, 2002; Ziogas et al. 1997, Patel et al. 2012, and Day et al. 1995)

It was observed that SHAM inhibited mycelia growth as indicated by poor growth compared to the non-SHAM amended control plate. This is because SHAM may have a toxic, or inhibitory effect on mycelial growth. The change in the PH caused by the acid may also explain the reduced mycelial radial growth in SHAM mycelial control study. This matches a study where SHAM was reported to have an effect of inhibiting *Ptr* mycelia growth (Maclean et al. 2017).

In the spore assays evaluated without SHAM in microtiter and Petri plates amended with picoxystrobin, the observed growth and germination could be explained not necessarily by the presence of an insensitive reaction but also because the fungus *Ptr* could have used the alternative oxidase since SHAM that would curtail the effects of alternative oxidase had not been incorporated. This infers that further investigations involving the use of SHAM and impact on spore germination. Scholars and other stakeholders ought to note

that the insensitivity to strobilurins fungicides which occurs by inducing the AOX pathway, allowing ATP synthesis to continue by circumventing the cytochrome b site of QoI action is reported only in vitro and is not transferable to the field conditions (Tamura et al. 1999). Hence, the isolates that showed insensitivity will best be concluded about by testing them in the greenhouse and field conditions.

The information gained from this study will guide future sensitivity studies for *Ptr.* Practices that assist in the avoidance or delay of fungicide resistance development such as rotation of fungicide chemistries, applying at the recommended doses, applying at the appropriate timing should be used for sustainable use of fungicide products.

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Table 4.1: Fungicides active ingredients used for mycelial and spore assays while establishing the sensitivity of *Pyrenophora tritici repentis* to the fungicides.

S/N	Fungicide	Mode of action	Full dose	half dose	double dose
1	Picoxystrobin	Inhibits ATP production	1.7 ml/l	0.85 ml/l	3.4 ml/l
2	Prothioconazole + tebuconazole	Inhibits sterol biosynthesis	2.75 ml/l	1.4 ml/l	5.5 ml/l
3	Azoxystrobin + propiconazole	Mixed mode of action	4.46 ml/l	2.23 ml/l	8.92 ml/l

Table 4.2: Thirty Ptr isolates that showed growth in the potato dextrose broth amended with picoxystrobin without SHAM in the microtiter assays

ID	County	State	Year
GPDN16-024 (B)	Dickenson	Kansas	2016
GPDN16-026 D	Samnen	Kansas	2016
GPDN16-036(C)	Bowman	North Dakota	2016
GPDN16-037(D)	Slope	North Dakota	2016
7.36.12	Watertown	South Dakota	2017
GPDN16-044 ss2	Griggs	North Dakota	2016
14-044-P1	NA	South Dakota	2014
GPDN16-002 ss1	Noble	Oklahoma	2016
GPDN16-007 ss3	Alfalfa	Oklahoma	2016
GPDN16-034(D)	Sargent	North Dakota	2016
GPDN16-001 ss2	Noble	Oklahoma	2016
GPDN16-024 (B)	Dickenson	Kansa	2016
58-5-17	Brookings	South Dakota	2017
58-6-17	Brookings	South Dakota	2017
GPDN16-026 (1)	Samnen	Kansa	2016
17.36.3	Watertown	South Dakota	2017
GPDN16-030	Moccasin	Montana	2016
ptr hand 1	Hand	South Dakota	2017
GPDN16-002 ss2	Noble	Oklahoma	2016
GPDN16-007 ss6	Alfalfa	Oklahoma	2016
GPDN16-024 (D)	Dickenson	Kansas	2016
13-7-P2	NA	South Dakota	2013
12-3-P4	NA	South Dakota	2012
13-1-P3	NA	South Dakota	2013
13-1-P5	NA	South Dakota	2013
13-9-P7	NA	South Dakota	2013
13-8-P8	NA	South Dakota	2013
14-029-P3	NA	South Dakota	2014
14-029-P13	NA	South Dakota	2014
GPDN16-001 ss2	Noble	Oklahoma	2016

NA Not Available for isolates whose counties of collection could not be retrieved

Table 4.3: Analysis of variance table showing the effect of different rates of picoxystrobin on five *Ptr* isolates tested using the Petri plate mycelia bioassay

Source of Variation	Df[a]	Mean Sq	Pr(>F)
Treatment	3	134.4	<0.001
Isolates	4	2.46	<0.01
Days after incubation	1	77.4	< 0.001
Treatment: Days after incubation	3	5.97	<0.001

[a]Degrees of freedom

Table 4.4: Effect of picoxystrobin on the five *Ptr* isolates amended on three different fungicide concentrations to ascertain their sensitivity/insensitivity using mycelia

Days after Incubation	Diameter (cm)	
7 DAI		
Control	4	a
Picoxystrobin Half Dose	2	b
Picoxystrobin Full Dose	1	c
Picoxystrobin Double Dose	0	d
12 DAI		
Control	7	a
Picoxystrobin Half Dose	4	b
Picoxystrobin Full Dose	3	c
Picoxystrobin Double Dose	2	d

Values are 60 replications for two pooled runs. Treatments with the same letters are not significantly different from each other according to Fishers Least Significant difference ($P \leq 0.05$)

Table 4.5: Effect of fungicides and the three doses, half, full and double on spore germination of five *Ptr* isolates without salicylhydroxamic acid

Treatment	**Isolate/ Germinated spores (%)**									
	GPDN16-024		**GPDN16-026**		**GPDN16-036**		**GPDN16-037**		**7.36.12**	
Check	45	a	42	A	31	a	52	a	31	a
Picoxystrobin Full Dose	25	b	28	B	20	ab	36	ab	20	ab
Picoxystrobin Half Dose	23	b	22	B	16	b	20	bc	16	b
Picoxystrobin Double Dose	7	c	8	C	16	b	12	cd	16	b
Prothioconazole+Tebuconazole Double Dose	4	c	7	C	13	bc	8	cd	13	bc
Azoxystrobin + Propiconazole Full Dose	3	c	4	C	8	bc	5	cd	8	bc
Prothioconazole+Tebuconazole Half Dose	2	c	3	C	7	bc	3	cd	7	bc
Azoxystrobin + Propiconazole Half Dose	2	c	2	C	0	c	2	d	0	c
Prothioconazole+Tebuconazole Full Dose	0.8	c	0.5	C	0	c	0	d	0	c
Azoxystrobin + Propiconazole Double Dose	0	c	0	C	0	c	0	d	0	c

Values are least squared means of 20 replications. Variables with the same letters are not significantly different from each other according to Fishers Least Significant Difference test, ($P \leq 0.05$)

CHAPTER 5

5.0 Conclusions and recommendations

Seed treatment results showed considerable low disease severities in fungicide seed-treated plants and this proves that systemic seed treatment fungicides might have an added benefit in curbing down foliar diseases such as tan spot and stripe rust especially in the early stages of plant development. However, the primary purpose of fungicide seed treatment should be for protection against soil/seed borne pathogens that interfere with seedling emergence.

The use of microbial pesticides in the management of tan spot in wheat plants proved to be effective. Visible and statistical reductions in disease severities were registered in plots and greenhouse pots that were pretreated with microbial and biochemical substrates and later inoculated with *Ptr*. Likewise, the use of biopesticides might improve yield as was registered in the microbial and biochemical treated plots as compared to the untreated plots. Products namely; *Bacillus subtilis* QST713, *Streptomyces lydicus* D747 and *Bacillus amyloquafasciens* Wyec 108 were effective against tan spot and can be recommended for use in organic wheat production once these products are registered for wheat.

The fungicide sensitivity study presented a number of isolates that showed continued germination in the presence of fungicides for both mycelia and spore bioassays without SHAM. However, when SHAM was incorporated in picoxystrobin for both spore and mycelia germination the previously insensitive isolates were sensitive. Further screening involving more isolates is needed in order to confirm insensitivity to picoxystrobin fungicide.

Overall recommendations for future studies could focus on the evaluation of the different biochemical and microbial pesticides in varying environments and on different pathogens like stripe rust, powdery mildew, and leaf rusts. Further tan spot studies should solely be conducted on no-till practices so as to have a good source of inoculum. Seed treatment studies should be expanded to evaluate other wheat foliar diseases like powdery mildew and leaf rust. Including chemistries reach in the Methyl benzimidazole-2-yl Carbamate (MBCs) group will be an added advantage. Fungicide sensitivity studies would have to be rigorously conducted by testing several isolates using SHAM in strobilurins for conidia spore assays. A bioassay that allows optical density readings would make quantification easy. Another key recommendation would be assessing the resistance risks associated with systemic seed treatment active ingredients when used alone or in combination with foliar sprays.